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Sodium Ion Extrusion Systems of *Bacillus subtilis*

by

Jianbo Cheng

A dissertation submitted to the Graduate Faculty in Biomedical Sciences
in partial fulfillment of the requirements of the degree of Doctor
of Philosophy, the City University of New York

1996

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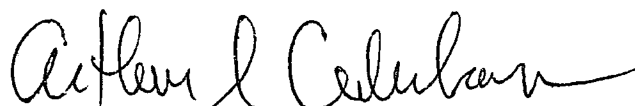

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Abstract

Sodium ion extrusion systems of *Bacillus subtilis*

by

Jianbo Cheng

Advisor: Professor Terry Ann Krulwich

Na^+ extrusion systems of *Bacillus subtilis* were studied by screening Tn*917*-mediated transpositional libraries. Mutant *B. subtilis* strains were isolated on the basis of growth inhibition by Na^+ at elevated pH. Two mutant strains, JC901 and JC111 were characterized in this study. Both strains exhibited a Na^+-sensitive phenotype at elevated pH, and were deficient in energy-dependent Na^+ extrusion. Efflux of $^{22}Na^+$ from whole cells was significantly reduced in the mutants upon re-energization. The capacity of the JC901 strain for pH homeostasis at pH 8.5 was unaffected relative to the wild type strain, while that of the JC111 strain was impaired. The site of transposition of JC901 is near the 3'-terminal end of a gene predicted to encode a membrane protein with six transmembrane spanning regions, designated *natB*. Together with an upstream gene *natA*, *natB* could function as an ABC type transport system. This system would belong to a subset of ABC type superfamily which are composed of only two types of polypeptides. A DNA fragment containing *natAB* cloned into an *Escherichia coli* vector enhanced the Na^+-resistance of an Na^+/H^+-deficient strain of *E. coli*. The insertional site of the transposon in JC111 was the promoter region of a gene which was previously identified by others as the *tetA*(L) locus. The gene product is a tetracycline efflux protein, which leads to pronounced

tetracycline resistance when the gene is present in multicopy, but the function of the single copy of *tetA*(L) that is normally present has been unclear. Studies of a *tetA*(L) gene deletion strain indicate that, in addition to resistance of a low concentration of tetracycline, the *tetA*(L) gene product in *B. subtilis* plays a significant role in Na^+-resistance, and a major role in Na^+- and K^+-dependent pH homeostasis at high pH. The cloned *tetA*(L) gene enhanced the Na^+-resistance of an Na^+/H^+-deficient strain of *E. coli*. To further characterize the *tetA*(L) gene product, a his-tagged *tetA*(L) gene was overexpressed in *E. coli*, and the gene product was purified and reconstituted in proteoliposomes. The purified preparation of TetA(L) cross-reacted with antibody raised against synthetic peptide corresponding to the N-terminus of TetA(L). The protein reconstitutes ΔpH-dependent transport of tetracycline, in the presence of cobalt, and of Na^+ in proteoliposomes. This strongly supports the conclusion that TetA(L) is a multifunctional antiporter and represents the first successful reconstitution of a Tet protein alone in proteoliposomes.

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List of Abbreviations

LB: Luria broth

Tris: Tris (hydroxylmethy) aminomethane

SSM: Spizizen's Salts-50 mM K-malate

TKM: Tris-50 mM K-malate

TTM: Tris-50 mM Tris-malate

ABC: ATP binding cassette

CCCP: carbonyl cyanide *p*-chlorophenylhydrazone

SDS: sodium dodecysulfate

EDTA: ethylenediamine tetra acetic acid

ONPG: *o*-nitrophenyl-β-D-galactoside

IPTG: isopropyl-1-thio-β-D-galactopyranoside

BSA: bovine serum albumin

TE: Tris EDTA buffer

TBS: Tris buffered saline

TTBS: Tween-20 + Tris buffered saline

PVDF: polyvinylidene difluoride

GCG: Genetics Computer Group

ORF: open reading frame

SD: Shine-Dalgarno

RBS: ribosome bindind site

NTA: nitrilo-tri-acetic acid

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Introduction

In most, if not all, living cells circulation of Na^+ ions is maintained across the cytoplasmic membranes. This circulation is generated by active Na^+-extrusion systems which extrude Na^+ and maintain an inwardly directed Na^+ gradient (20,28,97,99,113,124,125). The Na^+ gradient plays very important physiological roles in many bioenergetic processes, including Na^+-coupled substrate transport (69,73,79,106), and in some prokaryotes, Na^+-driven ATP synthesis (68) and flagellar movement (18,50). Maintenance of a low cytoplasmic Na^+ concentration also protects the cells from toxic effects of Na^+. The basis for the cytotoxicity of Na^+ is not completely understood. However, in several prokaryotes, including *Bacillus subtilis* (15,72) and *Escherichia coli* (97,99,100), Na^+ is more toxic at elevated pH values for growth than at neutral pH. This suggests that the toxic effects of Na^+ are due to its competition with H^+. The decrease of H^+ concentration may amplify the toxicity. In yeast (27) and *E. coli* (38), the K^+/Na^+ ratio is also a factor in the toxicity of Na^+ which is enhanced by either increase of Na^+ concentration or decrease of K^+ concentration. One target enzyme of Na^+ toxicity has been identified in yeast (89). Four mechanisms for Na^+ extrusion have been identified in bacteria, which include three types of primary extrusion and one type of secondary extrusion system. In the secondary extrusion systems, Na^+/H^+ antiporters, the electrochemical proton gradient that is established by respiration, proton-translocating ATPases, or other H^+ extrusion systems energizes an exchange of cytoplasmic Na^+ for H^+ (71,97,98). The three types of primary Na^+ extrusion systems that have been described in prokaryotes are: a V-type ATPase (64,127,134); Na^+ translocating decarboxylases that function in conjunction with certain anaerobic metabolic pathways (21); and

respiration-coupled Na^+ extrusion (6,40,139).

The current project was undertaken to enumerate and characterize the Na^+ extrusion systems of *Bacillus subtilis*, the most genetically developed experimental system and physiologically characterized Gram-positive bacterium. No prior studies of Na^+ extrusion or pH homeostasis in *B. subtilis* have been reported. We have screened *B. subtilis* Tn*917* transposition libraries for Na^+ and/or alkali-sensitive mutants. Five different strains were isolated, and two of them, designated, JC901 and JC111, have been studied intensively. The insertional site in JC901 appears to be a gene which is a part of an operon that encodes a homolog of the ABC-type transporters or traffic ATPases (3,46). This system plays a significant role in Na^+-extrusion, but not in Na^+-dependent pH homeostasis. It is also the first example of an ABC type transport system catalyzing inorganic cation extrusion. The transposon insertional site of the second mutant, JC111, is one previously identified as the *tetA*(L) locus of *B. subtilis* (2,94,117). The gene product is a tetracycline efflux protein, which leads to pronounced tetracycline resistance when the gene is present in multicopy. However, the function of the single copy of *tetA*(L) that is normally present has been unclear. Our experimental data show that the gene product does function in resistance to low tetracycline concentrations, but functions also as a Na^+/H^+ and probably a K^+/H^+ antiporter. The TetA(L) in *B. subtilis* plays a significant role in Na^+-resistance, and a major role in Na^+- and K^+- dependent pH homeostasis at high pH. This finding is of interest in connection with the origins of this class of resistance genes and their development, and with the mechanistic basis for a substrate flexibility.

Background

1) Prokaryotic Na^+/H^+ antiporters.

Na^+/H^+ antiporters catalyze the exchange of Na^+ for H^+. The existence of cation/H^+ antiporters was first proposed by Mitchell (86) and demonstrated in mitochondria by Mitchell and Moyle (87). In most aerobic prokaryotes (71,97-100), Na^+/H^+ antiporters are a major Na^+ extrusion system, driven by a H^+ gradient which is established by respiration-coupled proton extrusion.

The molecular research on Na^+/H^+ antiporters was initiated in *E. coli*. Two Na^+/H^+ antiporter encoding genes have been cloned by Padan and Schuldiner and their colleagues (67,104) and a third was cloned in our laboratory in collaboration with that group (58). They are *nhaA*, *nhaB* and *chaA*. The physiological roles of the gene products were investigated by deleting the genes from the chromosome. First, the phenotype of Δ*nha*A mutant (NM81) was tested (96). This strain could grow normally in low sodium medium at either low or high pH, but was inhibited, compared to wild type cells, when grown in high pH and high sodium medium. As the pH of media was elevated, strain NM81 showed increasing sensitivity to both Na^+ and Li^+. The Δ*nha*A strain still had Na^+/H^+ antiporter activity, although it was reduced about 50% compared to wild type cells. This led to the discovery of a second Na^+/H^+ antiporter-encoding gene in *E. coli*, designated *nhaB* (104). Studies of the consequences of a single deletion of *nhaB*, and of double deletions of both *nhaA* and *nhaB*, suggest that NhaB plays a role in extruding Na^+ during growth on substrates that are taken up together with Na^+. However, in the absence of NhaB,

NhaA alone can confer halotolerance (103). The H^+/Na^+ stoichiometries of the two Na^+/H^+ antiporters are different although they are both electrogenic (105,133). This has been determined by purifying the antiporter proteins, reconstituting them into proteoliposomes and determining the kinetics and steady state accumulation ration in relation to the driving force. NhaA is electrogenic and its H^+/Na^+ ratio is 2:1 (133), while NhaB is also electrogenic with a ratio of 1.5:1 (105). The sequence analysis revealed that both NhaA and NhaB are membrane proteins with 11 or 12 putative transmembrane-spanning regions linked with hydrophilic segments. The calculated molecular weights of NhaA and NhaB are, respectively, 41,316 and 55,534. The regulation of *nha*A was investigated by monitoring the β-galactosidase activity of a chromosomal translational fusion of *nha*A'-'*lac*Z that has been introduced into the gene coding region (66). The results showed that the gene could be induced by either NaCl or LiCl within an hour. Increasing the pH in the range of 6.5 to 8.5 without Na^+ does not enhance the expression of the gene, but the gene expression is markedly increased in the presence of either sodium or lithium. This inducibility of *nha*A raised the likelihood of the involvement of regulatory protein(s) in the expression of *nhaA*. Further sequence analysis of the *nhaA* region showed that there was another open reading frame downstream of *nhaA*, which is designated *nhaR* (109). It does not have its own promoter. The predicted amino acid sequence of NhaR suggested that it belonged to the LysR family of regulatory proteins with a N-terminal DNA binding motif, helix-turn-helix (44). The *nha*R deletion mutant strain was also constructed. The *nha*A and *nha*R double deletion strain had the same phenotype as the *nha*A deletion, but the single *nha*R deletion strain showed a difference compared to the *nha*A deletion. The single *nha*R deletion strain had the same phenotype as wild type when grown at pH 7.5. However, when the pH of the medium was elevated, the strain

started showing sensitivity to NaCl. Also when expressed in *trans, nha*R increased the Na^+ dependent expression of an *nha*A'-'*lacZ* protein fusion. A DNA binding assay indicated that NhaR specifically bound to the *nhaA* DNA fragment. Comparable studies of a *nha*B'-'lacZ fusion strain, showed that *nhaB* was expressed constitutively. The third antiporter of *E. coli* which catalyzes Na^+ and H^+ exchange at alkaline pH was first identified and characterized in our laboratory (58). In a functional complementation experiment, an *E. coli* gene (designated *chaA*) was cloned based on its ability to restore Na^+-resistance of NM81. Sequence analysis indicated that it was likely to be the structural gene for an antiporter. A region in one of the predicted hydrophilic loops of this protein was highly homolgous to the Ca^{2+} binding sites of other Ca^{2+} binding proteins. Everted membrane vesicles assays showed it possessed both Ca^{++}/H^+ and Na^+/H^+ antiporter activities. The gene was designated *chaA* and was proposed to be the structural gene for a $Ca^{2+}(Na^+)/H^+$ antiporter. Further experimental data from Kobayashi and his colleagues suggest that it plays an important role in Na^+ extrusion at alkaline pH (95). When the *chaA* gene was deleted from wild type strain, the Δ*chaA* mutant showed a Na^+ sensitive phenotype at pH 8.5, but the sensitivity was less than that of the *ΔnhaA* strain. The growth of the *nhaA* and *chaA* double deletion strain was completely inhibited by 0.1 M NaCl at pH 8.5. The *chaA* deletion strain also had lowered Ca^{2+}/H^+ antiporter activity at alkaline pH than that of wild type strain. This suggested that the gene product of *chaA* was also involved in Ca^{2+} extrusion at alkaline pH.

Na^+/H^+ antiporter-encoding genes in alkaliphilic *Bacillus* species have also investigated in our laboratory, and those of additional prokaryotes have been identified by other groups. Na^+/H^+ antiporters play a crucial role in extremely alkaliphilic *Bacillus* species. For them,

maintaining a cytoplasmic pH much more acidic than the outside is a central challenge. When the cells grow at pH 10.5, the antiporters electrogenically exchange extracellular H^+ for intracellular Na^+ so that the cells can maintain the cytoplasmic pH around 8.3 (73,74). The gene encoding one Na^+/H^+ antiporter (*nhaC*) has been cloned, by using a functional complementation protocol, and characterized in our laboratory (57). The Na^+/H^+ antiporter activity assay indicated that its product conferred Na^+/H^+ antiporter activity in cells of *E. coli* NM81 and the activity was stimulated by relatively high pH. A Na^+/H^+ antiporter-encoding was also cloned recently in another alkaliphilic bacterium, *Bacillus* speccies strain C-125 (36). A modest homology has been found between the two Na^+/H^+ antiporter-encoding genes from alkaliphilic *Bacillus* species. Genes encoding Na^+/H^+ antiporters were also cloned from another Gram-positive bacterium, *Enterococcus hirae* (142), and from two Gram-negative bacteria, *Salmonella entiritidis* (102) and *Vibrio alginolyticus* (90), by similar functional complementation approaches. Both genes from Gram negative bacteria have strong homology to *nhaA* from *E. coli*. The gene from *E.* hirae, designated *napA*, did not show significant homology to other Na^+/H^+ antiporters, although further sequence analysis suggested it might have some homology to an *E. coli* gene *kefC* encoding a K^+/H^+ antiporter (111).

2) Vacuolar-type Na^+-ATPase.

Vacuolar-type translocating ATPases were initially found in the vacuolar membranes of eukaryotic cells. The most common translocation substrate for this class of transporters is protons. Thus these ATPases function as proton pumps to acidify the vacuolar spaces. They are conserved among different species. Their structure is generally related to that of the F_1F_0-type

ATPases which are widely believed to be the evolutionary origin of V-type ATPases. V-type ATPases are large multisubunit complexes composed by two discrete parts. One is the large, hydrophilic, catalytic part (V_1) which can be released from membrane by washing with salt and the other is a hydrophobic integral membrane part (V_2) (26,132). The V-type H^+ ATPases are also found in archaebacteria and they are similar to the eukaryotic one (53,70). Recently, genes encoding a V-type Na^+ ATPase has been identified, characterized and sequenced in *Enterococcus hirae* (127,134). In *E. hirae*, two Na^+-extrusion systems, an Na^+/H^+ antiporter and an ATP-driven Na^+ pump, have been found (59,60,63,142). They have different roles in Na^+ extrusion. At neutral pH, Na^+ is driven primarily by the Na^+/H^+ antiporter using the electrochemical gradient established by a proton-translocating ATPase. At high pH (>9.5), the driving force of the Na^+/H^+ antiporter, the electrochemical proton gradient, is suggested to become too low to drive the extrusion of Na^+. The Na^+ extrusion is then mainly dependent on the Na^+-ATPase. The presence of an ATP-driven Na^+ pump in *E. hirae* was suggested by the work of Heefner and Harold (43). In an everted vesicles experiment, they showed the active uptake of Na^+ by everted membrane versicles, and the uptake was driven by ATP but was not inhibited by CCCP. The Na^+-ATPase was stimulated by both Na^+ and Li^+, but not by other ions. Furthermore, Kakinuma's group found that the catalytic part of this Na^+-ATPase could be released from membrane by EDTA treatment. Upon purification, it was found to be a 330 kDa protein complex composed of three subunits (73, 52 and 38 kDa). They partially sequenced the 73 kDa peptide. Based upon the peptide sequence, a series of PCR primers was generated to amplify the DNA coding region of the 73 kDa protein. A product of about 1 kb was amplified by a pair of primers and sequenced. The deduced amino acid sequence was compared to the gene bank. It showed strong homology to

some V-type H^+-translocating ATPases (61,65). In the biochemical studies of the Na^+-ATPase, they also found that it is highly sensitive to some V-type H^+-translocating ATPase inhibitors, like nitrate and N-ethylmaleimide (62). Based on both sequence analysis and biochemical studies, they proposed that this Na^+-ATPase was a V-type ATPase. With the sequence information from the PCR product, two DNA probes were made to screen the *E. hirae* cosmid library. Finally the whole operon was sequenced (134). In an independent study, Solioz and Davies also cloned and sequenced the operon by using a genetic approach (127). They first isolated a mutant strain by random chemical mutagenesis. The mutant strain, named NalkA, lacked a functional Na^+-ATPase and was not able to grow in the presence of more than 100 mM NaCl at pH 9.5, the condition at which Na^+-ATPase was required for keeping Na^+ out of cells and allowing optimal growth. The *E. hirae* plasmid library was transformed into the mutant strain. By the functional complementation of the mutant strain, they cloned and sequenced the operon of this V-type Na^+-ATPase.

3) Na^+-translocating decarboxylases.

Na^+-translocating decarboxylases function in some bacteria when they are grown anaerobically (21). A typical example is oxaloacetate decarboxylase in *Klebsiella pneumoniae*. In the early studies by Dimroth and colleagues, it was found that a novel metabolic pathway, with citrate as its substrate, was induced in *K. pneumoniae* when it was grown anaerobically on citrate as the sole carbon source. A membrane-associated, oxaloacetate decarboxylase is one of the major enzymes in this pathway. It catalyzes the decarboxylation of oxaloacetate which is generated from citrate lyase activity. This decarboxylation enzyme specifically needs Na^+ for

catalytic activity and it is inhibited by avidin, a biotin inhibitor (93,131). It has also been shown that in everted vesicles, this decarboxylation is coupled with electrogenic Na^+ uptake. By coupling active Na^+ extrusion to cellular metabolism of citrate, this enzyme converts the free energy from the oxaloacetate decarboxylation into a Na^+ gradient which is required for citrate uptake and pH homeostasis via a Na^+/H^+ antiporter (19,23). The decarboxylase consists of three different subunits, α(63.6 kDa), β(34 kDa) and γ(12 kDa). The α subunit contains biotin and it is a peripheral protein which can be dissociated from membrane by freezing and thawing in the presence of LiCl. Both β and γ subunits are integral membrane proteins. All three subunits have been purified and functionally reconstituted together into proteoliposomes (22). The cloning of genes encoding oxaloacetate decarboxylase was achieved by taking advantage of the fact that *E. coli* cannot grow anaerobically on citrate as the sole carbon source because it lacks the citrate metabolic pathway. A cosmid library of *K. pneumoniae* was transformed into *E. coli* and subsequently a few transformants which grew on citrate as the sole carbon source were isolated. The cosmids from these transformants were recovered. One cosmid expressed a Na^+-dependent citrate carrier and three subunits of oxaloacetate decarboxylase was subcloned into smaller plasmids. The genes encoding the citrate carrier and three subunits (γ, α and β) of decarboxylase were sequenced. They are, respectively, *citS, oadg, oada* and *oadb* and are arranged in the chromosome in this order (75,120,121,140,145). The *citS* genes could encode a very hydrophobic protein with 12 putative membrane-spanning helices. Based on the sequence analysis, the α subunit consists of two different domains. The N-terminal domain is homologous to other carboxyltransferases and is responsible for substrate binding, while the C-terminal contains a biotin-binding motif and is responsible for decarboxylation. Both *oadb* and *oadg* could encode

very hydrophobic proteins with several transmembrane segments. The γ subunit is predicted to contain one membrane spanning domain and the β subunit could contain either eight or nine. Upon both biochemical study and sequence analysis, a model has been proposed that the α subunit functions as a decarboxylase and the β and γ subunits function as a Na^+ transporter (21).

Similar decarboxylases have been found in other bacterial species, e.g. oxaloacetate decarboxylase in *Salmonella typhimurium* (146); methylmalonyl-CoA decarboxylase in *Propionigenium modestum* and *Veillonella alcalescens* (48,49); glutaconyl-CoA decarboxylase in *Acidaminococus fermentans, Peptostreptococus asaccharolyticus, Clostridium symbiosum* and *Fusobacterium nucleatum* (5,10).

4) Respiration-coupled Na^+ extrusion.

Respiration coupled Na^+-extrusion systems have been found in some marine and halophilic bacteria (139). Both of them need Na^+ for optimal growth and the requirement is probably due to the use of Na^+ as the ion for co-transport of amino acids (135). In the studies of the marine bacterium *Vibrio alginolyticus,* Tokuda and Unemoto found that a Na^+ gradient was initially generated by respiration even in the presence of a protonophore, CCCP. They suggested that a Na^+ pump was responsible for the formation of this Na^+ gradient. The extrusion of Na^+ by this Na^+ pump was inhibited by some respiratory inhibitors and activated by some electron donors. It was also independent of the cellular ATP concentration and had a relative higher activity under alkaline conditions (pH 8.5 to 9.0) (136). The coupling site of the Na^+ pump to the respiratory chain was then investigated by comparing the wild type strain to the mutant strains which were deficient in Na^+ pump activity. When an NADH:quinone reductase inhibitor HQNO (2-*n*-heptyl-

4-hydroxy-quinoline *N*-oxide) was used, it also inhibited the Na^+ pump. Together with the information, obtained in the early studies, that NADH:quinone reductase was stimulated by the presence of Na^+, the NADH:quinone reductase was then proposed to be the coupling site of Na^+ extrusion in the respiratory chain (137). Upon the initial purification of the reductase, it was found that this enzyme was composed of three subunits α, β and γ with molecular weights of, respectively, 52, 46 and 32 kDa. The β subunit contains FAD (flavin adenine dinucleotide) and catalyzes the reduction of ubiquinone to ubisemiquione. It shows NADH dehydrogenase activity by accepting electrons from NADH and reducing ubiquinone. The α subunit contains FMN (flavin mononucleotide) and is involved in Na^+ extrusion. The role of the γ subunit is to increase the affinity of the β subunit to ubiquinone (39,41,42). The genes encoding NADH:quinone reductase were subsequently cloned and sequenced by two groups (6,40), using a similar approach, i.e. using a series of oligonucleotides generated from the protein sequence to amplify the DNA coding region of the genes by PCR. A DNA probe was obtained from PCR for screening the genomic DNA library of *V. alginolyticus*, and then the whole operon was sequenced. Six open reading frames have been found in the operon. Three of them are found to encode subunit α, β and γ. The other three deduced gene products are very hydrophobic. Since hydrophobic proteins run poorly on SDS-PAGE gels, it is possible that these subunits were present but undetected in the purified samples that earlier showed activity. Or, perhaps the enzyme activity could be partially reconstituted by three hydrophilic subunits even without the hydrophobic subunits. Based on sequence analysis of the α subunit encoding gene *nqrF*, a model of Na^+ extrusion is established by Rich and his colleagues (112).

5) ABC type transporters.

ABC (ATP binding cassette) type transporters or traffic ATPases (3,46) are a superfamily of transport proteins which utilize the energy of ATP hydrolysis to actively transport various substrates against a concentration gradient. The most characteristic feature of this superfamily is the highly conserved ATP binding motif. ABC type transporters have been found extensively in both eukaryotic and prokaryotic cells. Some ABC transporters are extrusion systems which pump substrates from the cell, while others are uptake systems which accumulate substrates in the cell. The substrates carried by ABC type transporters are tremendously diverse and include sugars, amino acids, peptides, ions, oligopeptides, ploysaccharides and hydrophobic drugs. The molecular structure of ABC type transporters is a so called four membrane-associated domains (52). The four domains may be on separate and distinct proteins or in combinations on one or more proteins. Two of these domains are very hydrophobic and normally contain six transmembrane segments each. The hydrophobic domains form the pathway for substrates and determine the specificity to different substrates. The other two domains are hydrophilic and located in the cytoplasmic side of the membrane. The strong homologous sequences of the ATP binding motif have been found among these hydrophilic domains, which shows that they are involved in ATP hydrolysis (141). For individual domains which are formed by separate peptides, they could be either a homodimer or heterodimer. In some cases, another domain is also found, e.g. a periplasmic binding protein for many prokaryotic uptake system of the ABC type. Based on the sequence relatedness, a subfamily of bacterial ABC type transporters, ABC-2, has been established (110). The members of this subfamily function as export systems and most of them consist of two genes. One gene encodes a hydrophobic membrane protein, while the other

encodes an ATP-binding protein. Both of them are posited to form a homodimer to achieve the four domain structure. In some cases, two individual genes encode two different membrane proteins. So a heterodimer of the membrane domains is found in these cases. The substrates of the ABC-2 transporters vary from drugs to carbohydrates.

We report here a two gene system involved in Na^+ extrusion by *B. subtilis*. It apparently belongs to the ABC-2 subfamily. This is the first example of an ABC-type transporter which is involved in monovalent cation efflux. It is thus a new Na^+-extrusion mechanism, adding to categories of Na^+-extrusion systems described in bacteria. It functions as an electrogenic Na^+ pump and plays an important role in Na^+-resistance when the cell is exposed to an alkali environment or to membrane permeablizing solvents at neutral pH.

6) *tet* genes encoding tetracycline efflux proteins.

Tetracycline is a broad-spectrum antibiotic which is used for the treatment of infections caused by both Gram-positive and Gram-negative bacteria. It inhibits bacterial protein synthesis by binding to the 30S ribosomal subunits and preventing aminoacyl tRNA from binding to the subunit. Bacterial resistance to tetracycline is mediated by three different mechanisms: the active transport of tetracycline out of the cell (77,83); the protection of ribosomes from the binding of tetracycline (11,12); and the modification of tetracycline (129,130).

The most common resistance determinants belong to the active transport group (76). Early studies by Levy and colleagues (77,83,84) indicated that the members of this group possess an active efflux system which keeps the intracellular tetracycline concentration below the inhibitory level. Based on the different bacterial origins and gene structures, this type of

tetracycline resistance determinant has been grouped into different classes. In Gram-negative bacteria (37,82,85), all the resistant genes and their loci share some common features. The loci usually have a structural gene and a repressor gene, named respectively *tetA* and *tetR*. Those two genes are expressed divergently from overlapping operator regions. The regulation of the *tet* operon is controlled by the repressor protein. The presence of tetracycline will release the repressor protein from the promotor region of the structural gene and allow the transcription of the structural gene. A strong homology has been observed among the Gram-negative tetracycline resistance genes (8,47,92). The proposed secondary structure of this family is a 12 transmembrane segment model. A significant similarity has been found between each set of six transmembrane segments. It has been proposed by Paulson and Skurry that this 12 transmembrane segment protein has arisen as a result of a duplication of a ancestral 6 transmembrane segment protein (101,116). Genetic studies of the Tet proteins from Gram-negative bacteria have indicated that the each half of the protein with six transmembrane segments functions as separate domains. Both are required for tetracycline resistance. Combination of either two N-terminal halves or two C-terminal halves does not confer tetracycline resistance. But the combination of one N-terminal half and one C-terminal half from different classes of Gram-negative origin could form a active protein.(114,115). Yamaguchi and colleagues proposed that the substrate of the TetA protein was a divalent cation-tetracycline chelation complex (149). The stoichiometry of cation to tetracycline to protons was determined to be 1:1:1. Because the ionic form of tetracycline has one negative charge, the net charge change of the transport is zero, or the TetA is proposed to be a electroneutral antiporter (147).

The second family of active tetracycline transport systems is found in Gram-positive

bacteria. This family includes Class K, L and a recently cloned Class P (9,51,126). The genes of this family possess some different features from those in the Gram-negative family. There is no *tetR* gene found in the vicinity of the structure genes of this family, which means that the regulation of this family is probably via a different mechanism or set of mechanisms. The proposed regulatory mechanism is translational attenuation (122), a similar mode to that found for some other antibiotic genes such as *cat* and *erm* (25,80). No experimental data has been reported to show that this mechanism actually functions in regulation of the Gram-positive *tet*. There are, in fact, some indications of tetracycline-induced increases in *tet* mRNA for some of the Gram positive plasmid genes (88). The secondary structures of Gram-negative and Gram-positive *tet* gene products are also different. On the basis of hydropathy analysis, the secondary structures of Gram-positive Tet proteins were proposed to contain 14 transmembrane spanning segments (101). This model is different from the 12 transmembrane segment model not only in the number of segments but also in their hypothesized origins. Both of them are proposed to have evolved from the same ancestral 6 transmembrane transporter. The 12 transmembrane membrane transporters are thought to have arisen as a result of a duplication of the gene encoding this ancestral six transmembrane segment protein (116). The 14 transmembrane segment family appears to have evolved from the same ancestral protein, since their first 6 transmembrane segments have strong homology to that of the 12 transmembrane segment family proteins. The extra 8 transmembrane segments, instead of duplication, were presumed to have been captured from unknown sources (101).

Most of the *tet* (K, L, P) genes were initially found in Gram-positive bacterial plasmids. The study of the function of Gram-positive bacterial Tet proteins is far from comprehensive

compared to their Gram negative counterpart. It has been shown that the gene products of some plasmid *tet* genes are involved in active tetracycline efflux (84,148). In the studies of inverted membrane vesicles prepared from an *E. coli* strain which expresses *tet*(K), Yamaguchi and colleagues showed that the *tet*(K) gene product also functions as a metal-tetracycline/H^+ antiporter. In recent studies, they purified the Tet(K) protein in *E. coli* and reconstituted it into proteoliposomes. The function of the Tet(K) was observed only when the F_1F_0 H^+-translocating ATPase was co-reconstituted into the proteoliposomes (128). The chromosomal *tetA*(L) gene of *B. subtilis* is the first, and so far only, tetracycline resistance gene found in the chromosome. Recently, a gene which is homologous to Gram positive *tet* genes has been found in *Bacillus cereus* and the function of this gene is being investigated in our laboratory (*unpublished data*). The chromosomal *tetA*(L) gene of *B. subtilis* exists as a single copy near the origin of replication, but all the strains which contain this gene are nonetheless tetracycline-sensitive (2). The gene locus was first recognized by Williams and Smith in a mutagenesis experiment (143). A mutation induced by a chemical mutagen resulted in a tetracycline-resistant phenotype. It was proposed that the mutation changed the membrane permeability to tetracycline and the locus was named *tetB*, which should be *tetA*(L) according to the nomenclature proposed by Levy *et al.*(78). The gene was cloned by two different groups (54,117,118,123). They found that a fragment of the *B. subtilis* chromosomal DNA around the origin of replication could confer tetracycline-resistance when the fragment was inserted into a plasmid which did not possess its own tetracycline-resistance gene. The subsequent sequence analysis showed that the gene encoding a tetracycline efflux protein was very similar to plasmid *tet*(L) genes. It was proposed to be silent when present as a single copy in the chromosome. However, when the cells were treated in way that may have

led to more than a single *tet* gene copy, the gene could be amplified to multi-copy. It then conferred the resistance to tetracycline (54,55,144). The single copy gene was suggested by Ives and Bott to play an important physiological role, even although it did not confer resistance to tetracycline, because their attempts to delete the gene were unsuccessful (56). The real function of this gene has remained unclear. Our data now indicate that the *tetA*(L) gene product functions as a $Na^+(K^+)/H^+$ antiporter and plays a significant role in both Na^+-resistance and pH homeostasis. Although some Tet proteins from both Gram-negative and positive bacteria have been implicated in K^+ transport (24,30,31,33,91), none of them had been connected with Na^+ or K^+ efflux in exchange for protons. Our intriguing finding is of interest in connection with the origins of this class of resistance genes and with the growing list of multifunctional, multi-substrate transporters.

Materials and Methods

1) Bacteria strains and growth conditions.

B. subtilis BD99, obtained from Anthony Garro, is used as the wild type in this study. All the *B. subtilis* strains are routinely grown in either Spizizen salts medium (SSM) (86) or Tris medium, at 30°C with shaking. The Tris medium consists of 100 mM Tris-HCl buffer (pH 7.0 or 8.3), 1 mM potassium phosphate, 0.01% $MgSO_4$, 0.2% $(NH_4)_2SO_4$, 50 μg/ml each of threonine, histidine and tryptophan, 0.1% yeast extract and 50 mM Tris malate or potassium malate. When Tris malate is used, the medium is designated TTM. When potassium malate is used, the medium is designated TKM. When required, unless indicated specifically, antibiotics are used at the following concentrations for *B. subtilis*: chloramphenicol, 8 μg/ml; kanamycin, 5 μg/ml and tetracycline, 5 μg/ml. Tn*917*-mediated transpositional libraries from BD99 strain were prepared in earlier work in our laboratory by using plasmid pLTV1, which contains a derivative of Tn*917* (13). pLTV1 was transformed into the wild type strain. For each individual library, a single transformant was selected and then grown to large volume at 48°C in the presence of different antibiotics to kill the plasmid. The cells were harvested and stored at -70°C. Several libraries were prepared and used in previous studies (107,108). The isolation of transpositional mutants which were sensitive to high Na^+ concentration was carried out by replica plating. The libraries were plated on SSM prepared at pH 7 and replicated onto the same medium prepared at pH 9 and supplemented with 0.3 M NaCl. The strains which were negative for growth on the latter medium were selected. Each was tentatively presumed to be a transpositional mutant upon

being shown to lack the vector, pLTV1, marker but carry the chloramphenicol-resistance marker of the transposon. Totally, five such strains have been isolated and two of them (JC901 and JC111) were characterized in this study. Southern analysis of *Eco*RI-digested chromosomal DNA from all five strains, using a probe to the *cat* gene of Tn*917* (107), was performed before any further study to confirm that all of them possessed only a single transposon insertion (*data not shown*). JC112 is the BD99 strain with *tet*A(L) deletion constructed in this study.

E. coli DH5αMCR (GIBCO-BRL) was used in determinations of the sequence of the insertional site, and for cloning. It is also used in the overexpression of the *tetA*(L) gene with hexahistidine. *E. coli* EP432, a strain in which the Na^+/H^+-encoding *nhaA* and *nhaB* genes were disrupted (103) and *E. coli* NM81, a strain carrying a deletion in nhaA (96), were obtained from Etana Padan, and used for the functional complementation studies. The *E. coli* strains were usually grown in LB at 37°C, with 100 μg/ml of ampillicin added when appropriate; for *E. coli* EP432 and NM81, the NaCl in LB was replaced with KCl (LBK) (29), and kanamycin was added (50 μg/ml).

2) Analyses of the insertional site in JC901 and JC111.

The flanking regions of the insertional site in JC901 were cloned using the strategy developed by Camilli *et al.* (13), as described in connection with prior use of the same transpositional libraries (107,108) and as shown in Figure 1. Chromosomal DNA from both strains were digested with *Hpa*I and *EcoR*I, and the flanking regions of the transposon together with partial sequence of Tn*917* were self-ligated, creating plasmids pHPA901; pEC901 for JC901 and pHPA111 for JC111. The nucleotide sequences of chromosomal DNA in pHPA901, pEC901

and pHPA111 were determined initially by using primers to the known transposon sequence (P1--CTCACAATAGAGAGATGTCACCGTCAAG, P2--GGCGTATCACGAGGCCCT, P3--GTCT GCTCATCCATGACCTG and P4--TGGTTTAGTGGGAATTTGTACC). After the partial sequences of flanking regions were obtained from these primers, other synthetic oligonucleotide primers were then prepared commercially (Genset) based on the known sequences. An Applied Biosystems 373A automated sequencer in the DNA Core Laboratory of the Mount Sinai School of Medicine was employed for the sequencing reactions. Routine molecular biological procedures were carried out as described by others (4,119). DNA sequence analyses were performed on a GCG program (17) on VMS system.

3) Cloning of the chromosomal *natA*, *natB* and *tetA*(L)genes.

The *natAB* and *natB* genes were amplified by PCR from chromosomal DNA from the wild type strain of *B. subtilis*. Two possible versions, assuming two different translational start sites, were prepared for *natB*. The pairs of primers (shown schematically in Figure 5) used for the PCR reactions were: F0--AAGAACTCTTCGCGACAAC and R0--AATCCGATCCGCTTCAC for pJN1; F1--GGAGGAAGTGCAGGCGCT and R0 for pJN2; and F2--GCAGTCTGCCTTAG ATCAG and R0 for pJN3. The amplified products were gel-purified and blunt end-ligated into the *Hinc*II site of pGEM3Zf(+). This produced the three plasmids designated pJN1, pJN2 and pJN3 which had, respectively, the whole *natA* and *natB*, the "large" version of *natB*, and the "small" version of *natB* cloned under control of the T7 promoter. They were prepared in *E. coli* DH5αMCR and subsequently transformed into *E. coli* EP432. To prepare a recombinant vector which could express *natAB* genes in *B. subtilis*, the fragment contained both *natA* and *natB* were

released from pJN1 by digesting the plasmid with *Xba*I and *Pst*I. This fragment was then ligated into the multiple cloning site of a shuttle vector, pYH56, which was constructed by and obtained from Dr. Bechhofer. pYH56 had been constructed with origins of replication from pUB110 and pGEM. The resulting plasmid, designated pJY1 was prepared in *E. coli* DH5α and subsequently transformed into both wild type and JC901 strains of *B. subtilis*.

The same PCR cloning strategy was also used in the cloning of the *tetA*(L) gene. The three primers used were: TF1--GGAGGGGATCCATGAATACGTCTTATTCACAG, TR1--TTTCACAAGCTTAGCCATGTCTCCGCGAACG and TR2--TTTCACGGATCCAGCCATGT CTCCGCGAACG. TF1 and TR2 were customized with a *BamHI* cut site and TR2 was customized with a *Hind*III cut size. The first construct was made by cloning the PCR product amplified by using TF1 and TR1 into the *HincII* site of pGEM3Zf(+). The resulting plasmid, designated pJTA1, contains the whole coding region of the *tetA*(L) gene under control of the T7 promoter. To express the *tetA*(L) gene in *B. subtilis*, another construct was made from the same PCR product. After the product was double digested with *Bam*HI and *Hind*III, and gel-purified, it was then ligated into the multiple cloning site of pBK36 which was also double digested by *Bam*HI and *Hind*III. The resulting plasmid, designated pTL1, contains the whole coding region of the *tetA*(L) gene under control of the *ermC* promoter. Plasmid pBK36 obtained from Kevin Zen was constructed with replication origins of pUB110 and pBR322. To purify the *tetA*(L) gene product in the *E. coli* strain, a construct of the *tetA*(L) gene was made in which a hexahistidine was attached to the C-terminal end of TetA(L). Two primers TF1 and TR2 were used to amplify the *tetA*(L) gene. The PCR product was digested with *BamHI*, gel-purified, then inserted into the *BamHI* site of plasmid pQE12 (Qiagen). The new construct, named pJQ2, with the *tetA*(L) gene

under control of the T5 phage promoter of the plasmid was selected. The expression of the modified *tetA*(L) gene (with an extra six histidines encoded the 3' end) was under control of two *lac* operator sequences.

4) Na^+ flux experiments.

Cells were grown in SSM medium at pH 7 to mid-logarithmic phase, were harvested by centrifugation, then washed by 100 mM Tris buffer pH 8.5 and suspended in the same buffer to 20 mg protein/ml. Cell protein was determined by the method of Lowry *et al.* (81), using egg-white lysozyme as the standard. The cells were passively loaded with $^{22}Na^+$ by incubation with 5 mM $^{22}NaCl$ at 21°C for two hours. Since there was no "food" available for the cells in the buffer, the energy level of the cells was low or the proton motive force of the cells was relatively lower. The consequence of the low energy state was the accumulation of Na^+ inside of the cells. For the experiments in which $^{22}Na^+$ efflux was driven by respiration, 2 µl of cells was diluted at 21°C into 400 µl of 100 mM Tris buffer, pH 8.5, containing 10 mM Tris malate. In potassium diffusion potential driven $^{22}Na^+$ efflux experiments, valinomycin was added in the cell suspension at a final concentration of 1 µM. 2 µl of cells was diluted at 21°C into 400 µl of either 100 mM Tris buffer, pH 8.5 or 100 potassium phosphate buffer, pH 8.5, both of which contain 1 µM of valinomycin. Samples were taken and filtered directly via a Millipore filtration apparatus, and washed with 5 ml of the same dilution buffer. All the filters were air dried and their radioactivity was counted by scintillation spectrometry. Numbers presented are the average of at least four independent experiments.

5) Na^+ accumulation experiments in *E. coli* strain EP432.

The concentration of cytoplasmic Na^+ in various transformants of *E. coli* EP432 was determined when they were grown in LBK + 350 mM NaCl at pH 7.0, 37°C. In each experiment, 250 µl of an overnight culture for each transformant was inoculated into four flasks containing 25 ml of medium. $^{22}Na^+$ was added to two flasks of each set (4 µCi/ml) and the other two flasks were used in parallel for determinations of the A_{600} and protein concentration. When the cells reached A_{600} = 0.3~0.5, CCCP was added, to a final concentration of 50 µM, to one of the radioactive and one of the non-radioactive flasks. Although at this concentration, CCCP might not completely deenergize the cells, it did completely stop the growth of the cells. After an additional hour of growth, six 1 ml samples were taken from the two radioactive cultures. These samples were directly filtered and washed with 8 ml of non-radioactive medium. A second set of samples were treated with 5% butanol (vortexing for 30 seconds) before filtration and washing. All the filters were air-dried and their radioactivity was counted by scintillation spectrometry. The cytoplasmic $^{22}Na^+$ cpm from each set were determined by subtracting the cpm from the butanol treated cells from the original counts. The Na^+ concentration was calculated using the cytoplasmic $^{22}Na^+$ cpm from the CCCP-treated cultures. The latter values were assumed to represent the internal Na^+ concentration at equilibrium with the external Na^+ concentration (350 mM). There maybe a consistent, small error if the CCCP-treated cells were not completely de-energized, e.g. because of CCCP extrusion by the cells. Values presented were the averages of all the samples in at least three independent experiments.

A second protocol was used to compare the CCCP-sensitivity of the $^{22}Na^+$ extrusion from EP432 strain driven by NatAB and *E. coli* NhaA. Both pGM36 and pJN1 transformants were

grown, in duplicate, in 20 ml of LBK containing $^{22}NaCl$ (0.35 M, 4 μCi/ml) and 1% glucose, pH 7.0, at 37°C, to A600 of about 0.7, and then the culture was divided into two separate tubes. To one tube, CCCP was added to a final concentration of 50 μM. After an additional 5 min of incubation, six 1 ml samples from each tube were filtered, washed, and counted as described above. Values averaged from four independent experiments are presented.

6) β-Galactosidase assays.

For the induction of β-galactosidase in JC901, various additions were made to TTM growth medium 20 μl of overnight culture was inoculated into 2 ml of medium in a 15 ml conical tube at 30°C with shaking for 15 hours. The β-galactosidase activity was determined essentially as described by others (32). Culture were harvested by centrifugation and washed twice with 500 μl of β-galactosidase assay buffer: 100 mM sodium phosphate (pH 7.0), 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol. The final pellet was suspended in 200 μl of β-galactosidase assay buffer, 20 μl of toluene was added, and the cell suspension was vigorously vortexed for one minute. The toluene was evaporated off by placing the cell suspension in a 37°C water bath for 15 minutes. An additional 600 μl of assay buffer was added, and the suspension was incubated 5 minutes at 30°C. The β-galactosidase assay was initiated by the addition of 200 μl of 4 mg/ml o-nitrophenyl-β-D-galactoside (ONPG). The assay proceeded at 30°C for various times and was stopped by the addition of 500 μl of 1 M Na_2CO_3. Samples were centrifuged to remove debris, and the absorbance of the supernatant (A420) was determined spectrophotometrically. β-galactosidase activity was expressed as μmole ONPG hydrolyzed/min/mg of protein.

7) Targeted deletion of the *tetA*(L) gene from wild type BD99.

The details of cloning strategy are illustrated in Figure 2. Briefly, a 4.5 kb *Hind*III chromosomal DNA fragment from pCIS7 (54) which contains the *tetA*(L) gene and surrounding regions was cloned into the *Hind*III of pGEM9Zf(+) (Promega) and that resulted in plasmid pJBC1. The *cat* gene from pC194, cloned in pSCD2 as an *Mbo*I fragment (constructed in other study), was released by digestion with *HpaI* and *NaeI*. It was then ligated into the *Stu*I site of pSE420, resulting in plasmid pJBC2. The next step was to replace the *Cla*I-*Nde*I fragment of pJBC1 which contains the *tetA*(L) locus with the *Cla*I-*Nde*I fragment from pJBC2 containing the *cat* gene. That resulted in a new plasmid pJBC3. pJBC3 was linearized by digestion with *Afl*III and then transformed into wild type BD99 strain. Chloramphenicol resistance was used as a selective marker for the *tetA*(L) deletion strain, designated as JC112. Southern analysis of *EcoR*I-digested chromosomal DNA from JC112, using both *tet* and *cat* probes, confirmed the replacement of the *tetA*(L) gene by the *cat* gene.

8) Northern analyses and determination of the transcriptional start sites.

To isolate RNA, 20 ml of logarithmic phase cells (A600 ≐ 0.6) growing in TTM pH 7.0, were harvested by centrifugation and suspended in 4 ml of protoplasting buffer (15 mM Tris, pH 8.0, 6 mM EDTA, 0.45 M sucrose and 2 mg/ml lysozyme) . After 20 min of incubation at 4°C, the cells were pelleted and resuspended in 2 ml of β-mercaptoethanol buffer (80 mM Tris pH 8.5, 10 mM $MgCl_2$ and 10 mM β-mercaptoethanol). 2 ml of proteinase K buffer (500 μl/ml proteinase K , 1% SDS, 20 mM EDTA, 0.8 mg/ml 1-10 phenanthroline and 0.4 mg/ml Na-heparin) were

added. The cells were incubated at 37°C for 30 minutes and then passed through a 17½ needle twice. After phenol/chloroform extraction and ethanol precipitation, the samples were dried in a speed vacuum. The pellets were suspended in 200 µl of DNase buffer (100 mM NaOAc and 5 mM $MgSO_4$) and then 2 U of RQ1 DNase (Promega) was added. After 15 minutes of incubation at 37°C, the samples were subjected to another phenol/chloroform extraction and ethanol precipitation. Final samples were resuspended in 50 µl of TE (10 mM TrisCl pH 7.5 and 1 mM EDTA). The RNA concentrations were determined by reading $A_{260/280}$. For Northern blot analyses, 10 µg of each RNA sample was run on a 0.8% agarose gel and blotted onto a nylon membrane (Schleicher & Schuell) overnight. Two RNA probes were used for hybridization (shown in Figure 13). One probe (R1) was prepared from a construct which contains the *tetA*(L) gene coding region *Pst*I fragment. The other (R2) was prepared from pJBC3 that was linearized with *Nde*I. *In vitro* transcription with T7 (for R1) or SP6 (for R2) RNA polymerase was carried out essentially as described by Promega, and the products were labelled with ^{32}P-α-UTP.

Reverse transcriptase analysis was used to determine the transcriptional start site(s) the *tetA*(L) gene as described by others (7). Fifty µg of RNA prepared from both the wild type strain and JC111 containing pCIS7 (JC111+) was used. An oligonucleotide primer which was complementary to a sequence at the 3' end of the leader region (shown in Figure 14) was used in the reaction. The same primer was also used for dideoxy sequencing of pCIS7. The primer used in the reverse transcriptase reaction was first labeled with ^{32}P-γ-ATP at the 5' end by using T4 polynucleotide kinase (USB). After a total of 50 µg of RNA was denatured at 65°C for 10 minutes in 14 µl of reaction mixture (50 mM Tris pH 8.0, 8 mM $MgCl_2$, 30 mM KCl and 1 mM DTT), 5 ng of labeled primer was added. The sample was incubated at 42°C for another 4 minutes and then deoxynucleotide triphosphates were added to a final concentration of 0.5 mM. The

reaction was started by adding 12 U of avian myeloblastosis virus reverse transcriptase. After 30 minutes of incubation at 42°C, the reaction was stopped by adding EDTA to a concentration of 50 mM. The sample was extracted with phenol/chloroform and precipitated with ethanol. The dried pellet was resuspended in 4 μl of DNA sequencing loading buffer, heated to 80°C for 2 minutes, and then run on an 8% polyacrylamide-urea sequencing gel along with the sequencing ladder of pCIS7, using the same unlabeled primer. The sequencing protocol was as described by UBS.

9) Overexpression and purification of TetA(L).

Plasmid pJQ2 was used for overexpressing the *tetA*(L) gene, and the *E. coli* DH5α strain was used as the host. The whole system for overexpression and purification was describe by Qiagen Inc. To avoid the toxicity of the gene product to the host, plasmid pREP4 which contains a *lac*Iq gene was first transformed into *E. coli* DH5α. A transformant was selected, to which pJQ2 was transformed. The new transformant, contains both pREP4 and pJQ2. To get maxim inhibition the gene expression, 2% glucose was routinely added to LB medium. In the initial purification, 5 ml of overnight culture was inoculated in 1 liter of LB glucose medium. After grown, at 30°C, to an A_{600} of about 0.7, the cells were pelleted by centrifugation and resuspended into a same volume of fresh LB containing 2 mM IPTG. The cells were grown for two more hours at 30°C and then harvested by centrifugation. The pellets were kept at -20°C. The preparation of cell membranes was described in section 5. The final membrane pellets were resuspended in 200 μl of extraction buffer (20% glycerol, 20 mM Tris-HCl pH 8.0, 600 mM NaCl and 0.25 mg/ml asolectin). After extraction with 1% lauryl-maltoside, the sample were

ultracentifuged at 150,000 x g for one hour. The supernatant was collected and mixed with 50 µl of Ni-NTA resin by nutaing at 4°C for one hour. The proteins bound to resin were eluted with 0.1 M EDTA. 20 µg of such protein samples were then run on a 10% SDS-polyacrylamide gel and the protein bands were visualized by either Coomassie staining or Silver staining. One such gel (before staining) was transferred onto a PVDF membrane (Bio-rad) and the TetA band was isolated and sequenced in the Protein Chemistry Core Laboratory of Mount Sinai School of medicine. The sequence data confirm that the band was derived from TetA. The first six N-terminal amino acids of the purified protein are SQSTLR, which represent amino acids # 10 - 15 of the deduced N-terminal sequence. Four amino acids from the vector and five from the *tetA*(L) gene were missing. In attempt to maximize the yield, seven different detergents were used to extract the TetA(L) protein. Lauryl-maltoside was found to give the best extraction (*data not shown*).

For large scale purification of Tet(A).protein, a total 100 mg of membrane protein mixture was resuspended in 10 ml of the extraction buffer. After lauryl-maltoside was added to a final concentration of 1%, samples were incubated at 4°C with occasional shaking for 1 hour. Samples were ultracentifuged at 150,000 x g for one hour and the supernatant was mixed with 1 ml of Ni-resin, which was equilibrated with column buffer (20% glycerol, 20 mM Tris pH 8.0 and 0.04% lauryl-maltoside) + 10 mM imidazole, by nutating at 4°C for 1 hour. Samples were then transferred in a column. The column was washed, first with 40 ml of column buffer containing 10 mM imidazole, and then 5 ml of the same buffer containing 20 mM imidazole. The Tet protein was eluted with 10 ml of the same column buffer containing 100 mM imidazole.

10) Western immunoblot analyses.

To raise an antibody against TetA(L), a 14-mer oligopeptide corresponding to the deduced N-terminal sequence was synthesized in the Protein Chemistry Core Laboratory of Mount Sinai Medical School. The first amino acid residue (from N-terminal) was switched from valine to cysteine, since cysteine was required for the conjugation of this peptide with immuno-stimulating protein. The conjugation was carried out by using maleimide-activated Keyhole Limpet Hemocyanin as described by Pierce. This conjugated antigen was then injected into a rabbit using the facilities of the Center of Experimental Animals of Mount Sinai Medical School. Blood samples was collected at different times.

5 μg of protein samples from the initial Ni-resin and EDTA purification were run on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-rad). The membrane was soaked in 100 ml of in TBS (0.8 % NaCl, 0.038% KCl, 0.0015 % phenol red and 0.3% Tris at pH 7.4) containing 3% BSA for 1 hour, and was then washed with TTBS (TBS with 0.05% Tween-20). The membrane was incubated overnight with a blood sample from the rabbit (1:100 dilution in TTBS containing 1% BSA). The color was developed by using alkaline phosphatase protocol (Bio-rad).

12) Reconstitution of Tet protein into proteoliposomes.

First an asolectin suspension was prepared by adding 1 ml of a buffer containing 100 mM MOPS, pH 7.0; 1 mM DTT and 1.2 % octylglucoside to 40 mg of asolectin and sonicating in a bath type sonicator until clear. 150 μl of the asolectin suspension was then mixed with 450 μl of the purified protein (total about 40 μg), to which octylglucoside was added to a concentration

of 1.2 %. After being sonicated briefly, the mixture was diluted 50 fold into a dilution buffer (1 mM DTT, 150 mM NH_4Cl and 15 mM Tris, pH 7.0) and mixed by stirring for 20 minutes at room temperature. Proteoliposomes were collected by centrifugation at 150,000 × g for one hour and resuspended in 100 µl of the dilution buffer. ΔpH-driven $^{22}Na^+$ uptake was initiated by dilution of 4 µl of proteoliposomes in to 500 µl of 150 mM choline chloride or NH_4Cl (control without pH gradient), 15 mM Tris, pH 8.5, and 1 mM $^{22}NaCl$ (4 µCi/µmole). At intervals, the reaction was stopped by filtering samples onto a 0.22-µm-pore-size filters (Millipore), which were then washed with 2 ml of cold reaction buffer. Radioactivity of the filters was measured in a scintillation counter. Tetracycline uptake experiments were done practically in the same way except that 50 µM [^3H]tetracycline (20 µCi/µmole) and 100 µM $CoCl_2$ were added instead of $^{22}NaCl$. Numbers presented are the average of two independent experiments and in each experiment the values were determined in duplicate.

A Two Gene ABC-type Na^+ transporter system

Results:

1) Growth properties and Na^+ extrusion of JC901.

The Growth of JC901 and its sensitivity to added NaCl were compared to that of the wild type in SSM medium at pH 7 or 8.3. As shown in Figure 3, growth of the wild type and JC901 were indistinguishable at both pH 7 and pH 8.3 in the absence of added salts, with better growth of both strains observed at neutral pH. Although not shown, addition of NaCl (to 0.7 M) at pH 7 had no effect upon the growth of either strain. By contrast, at pH 8.3 addition of 0.7 M NaCl inhibited the wild type modestly and JC901 profoundly.

The Na^+-extrusion activity of JC901 strain was also tested. Whole cells of both wild type and the JC901 strain were starved and passively loaded with $^{22}Na^+$ by incubation with 5 mM radiolabelled NaCl for about 2 hours at pH 8.5. Cells were reenergized by the addition of malate. As shown in Figure 4, malate-dependent and independent efflux of $^{22}Na^+$ was observed from both JC901 and wild type. The over all $^{22}NaCl$ efflux from JC901 was significantly slower than that from wild type. Thus the Na^+-sensitive phenotype was accompanied by a partial loss of energy-dependent Na^+ efflux capacity. Subsequent experiments by Arthur Guffanti (14) have shown that the growth and transport phenotype of JC901 is complemented by pJY1, the construct of pYH56 into which I cloned *natAB*. In addition, using cells that were more completely depleted of energy than those shown in Figure 4, it was shown that $^{22}Na^+$ efflux by the pJY1-complemented strain was stimulated by 50 μM CCCP.

2) Sequence of the disrupted gene locus in the JC901 strain.

By using the strategy described in Materials and Methods, sequence data were obtained from both *EcoR*I and *Hpa*I fragments of the flanking region. As shown in Figure 5, the transposon was inserted into a putative operon. Three possible open reading frames, designated *natA*, *natB* and *orfC*, were found upon sequence analysis, although the sequence of *orfC* was not complete. Both *natA* and *orfC* are preceded by a ribosome-binding site. For *natB*, whose 3' end was disrupted by the transposon, two possible SD sequences and translational start sites are found and indicated in Figure 5. The nucleotide sequence of this region, which was deposited in Genbank™/EMBL with accession number U30873, is shown in Figure 6. A putative promotor sequence was found before the three open reading frames. Although about 2 kb of the upstream sequence of the promotor was also determined, the only apparent ORFs were predicted to be transcribed in the opposite direction from *natAB* and there was no evident relationship between those and *natAB*.

The deduced product of *natA* would be a hydrophilic 27.9 kDa protein. When compared to the gene bank data base using FASTA program of GCG, NatA presents a significant homology with a group of ATP-binding proteins of the ABC type transport systems (Figure 7). The most conserved ATP-binding regions, Walker A and Walker B, were found in the sequence. The best two matches of FASTA list were DrrA, a component of a drug efflux system from *Streptomyces peucetius,* and an ATP-binding protein from alkaliphilic *Bacillus firmus* OF4. The latter was cloned in our laboratory as part of *B. firmus* OF4 chromosomal DNA that functionally complimented a Na^+ sensitive *E. coli* EP432 strain (*unpublished data*). The identity between NatA and DrrA and ABP (ATP binding protein) are, respectively, 36.4% over a 206 aa region

overlap and 34.4% over a 189 aa region overlap (Figure 7). The *natB* gene could encode a hydrophobic protein with a molecular weight of about either 36.9 or 23.2 kDa depending on which of the two possible translational start sites was utilized. The hydropathy profile indicates that there are six transmembrane segments in the C-terminal of the protein (Figure 8). It could encompass most of the smaller protein product if the second translational start site was used. The sequence itself does not exhibit a strong homology to the prokaryotic protein sequences in the data base. However there is a modest similarity to the hydrophobic regions of some eukaryotic Na^+/H^+ antiporters. The region of similarity is shown for the best match (Figure 9), the rabbit Na^+/H^+ exchanger NHE3. The identity was 25.8% over a 128 aa region of overlap. The region of NHE3 involved encompassed its putative membrane-spanning segments 2-4, but not the most conserved 5a and 5b regions (138). The remaining N-terminal half of the larger version of the *natB* gene product was predicted to be hydrophilic and no significant similarity was found between it and any other sequences in the data base. On the other hand, extensive secondary structure is predicted for the RNA produced from the region between *natA* and the second possible *natB* start when estimated by the FOLD program. The third open reading frame of the flanking region is designated *orfC* and its complete sequence is not found in the cloned flanking region which was sequenced. The deduced amino acid sequence was compared to the gene bank data base. It shows modest sequence similarity to dehydrogenases and reductases that are involved in metabolism of a variety of plant products, especially alcohols and isoflavonoids.

3) Expression of *natAB* gene in a Na^+sensitive *E. coli* strain

The Na^+/H^+ antiporter-deficient strain of *E. coli*, EP432, was used to determine whether

expression of *natAB* gene would functionally complement the Na^+sensitive phenotype of such a strain. The plasmids pJN1, pJN2 and pJN3, were transformed into EP432 to examine: whether pJN1, containing the whole *natAB* sequence, would increase Na^+-resistance; and whether either of the *natB*-containing plasmids, which show modest sequence similarity to eukaryotic Na^+/H^+ exchangers, pJN2 and pJN3, might catalyze Na^+/H^+ antiport, and hence also functionally complement when expressed without *natA*.

In the initial test, the transformants were grown in LBK medium in the presence of 400 mM NaCl, the condition at which the growth of the EP432 strain is totally inhibited by NaCl. As shown in Figure 10, neither pJN2 nor pJN3 helped the growth of an *E. coli* EP432 transformant on LBK medium + 400 mM NaCl relative to the negative control, but pJN1 allowed growth that was almost as robust as that of a transformant with plasmid pGM36 which expresses the *E. coli* *nhaA* gene encoding a Na^+/H^+ antiporter. Interestingly, the *E. coli* EP432 transformants containing the two inserts with *natB*, pJN2 and pJN3, were more sensitive to Na^+ than the transformant with control plasmid when they were grown at sub-inhibitory Na^+ concentrations (200 mM NaCl), although no difference was observed on LBK medium in the absence of added Na^+ (*data not shown*). The phenotypic properties exhibited by the transformant with the larger version of *natB* (pJN2) are the same as those observed with the smaller version of *natB* (pJN3). Addition of *natA* gene product together with the *natB* gene product was required for the enhancement of Na^+-resistance. It is unlikely that *natA* gene itself would complement the resistance alone since the sequence data indicate that it is a hydrophilic ATP-binding protein.

To better document the mechanism of Na^+-resistance mediated by the *natAB* gene products, the cytoplasmic Na^+ concentrations of the *E. coli* EP432 transformants were

determined when they were grown in the presence of $^{22}NaCl$. As shown in Fig 11, transformation of *E. coli* EP432 by pJN1 resulted in enhanced growth in the presence of 350 mM added NaCl at pH 7.0, although the effects were not as great as those conferred by pGM36 . The growth pattern of the transformants in broth was consistent with that on the Petri-dishes. The growth data were also consistent with the measurements of internal Na^+ concentrations of the transformants (shown on the right of Figure 11). Both pGM36 and pJN1 led to a lower cytoplasmic Na^+ concentration, which correlated with improved growth of *E. coli* EP432. The lower the internal Na^+ concentration in the transformants, the better they grew. In most experiments the poor growth of *E. coli* EP432/pJN2 resulted in the lack of enough cell material to perform the measurement of internal Na^+. The data raise the possibility that the sole gene product of pJN2 may result in a Na^+-specific leak. The mechanism of the active Na^+ efflux mediated by the *natAB* gene products was studied by using the uncoupler CCCP. Na^+ extrusion by a secondary Na^+/H^+ antiporter would be inhibited by the uncoupler, whereas primary Na^+ extrusion would be stimulated as long as the uncoupler treatment was brief enough to preserve cellular ATP. *E. coli* EP432 transformed with pGM36 and pJN1 were grown in the presence of 350 mM $^{22}NaCl$. An inward Na^+ gradient was established by both transformants based on the Na^+ extrusion experiment data. As shown in Table III, a five minute treatment with 50 μM CCCP resulted a significant increase (16%) in the cytoplasmic Na^+ concentration in the pGM36 transformant, while it resulted a slightly greater Na^+ exclusion in the pJN1 transformant. Hence, the NatAB system apparently does not directly use a proton gradient as the energy source, nor is NatAB-mediated Na^+ extrusion coupled to proton uptake. These data support the results from the sequence analysis that the NatAB system is an ATP-driven primary Na^+ extrusion system and

the absence of an apparent role of the locus in pH homeostasis (14).

4) Expression of the *lacZ* gene of the inserted transposon in JC901 strain..

Sequence analysis showed that the insertion of the transposon in JC901 placed the promoterless *lacZ* gene in the same direction of transcription of *natAB* so that expression of the gene locus could be monitored by assaying β-galactosidase activity. A series of substrates was examined for their effects on the *natB* expression. As shown in Table IV, both NaCl and KCl increased the expression of the gene by 2-3 fold, while KCl induced slightly more than NaCl. CholineCl, at the same concentration, did not increase the expression. Increase of pH did not induce the expression, nor did it enhance the induction by NaCl. Interestingly, ethanol also resulted in a significant increase in the gene expression. Sucrose, at high osmotic strength, significantly decreased the gene expression. The results indicated that the cations were responsible for the induction of *natAB*, but not osmotic or ionic strength or alkali pH. Exposure of the cells to ethanol also could induce the expression of these genes.

Discussion:

In this study, we report a new primary sodium extrusion system, *natAB*, an ABC type transport system found in *B. subtilis*. Disruption of the *natAB* gene locus results in a sodium sensitive phenotype at elevated pH. The sodium extrusion ability of the mutant strain is considerably impaired compared to the wild type strain. From the DNA sequence analysis, *natA*

shows a strong homology to other ATP-binding proteins of ABC type transport systems. The universal ATP binding-sites, Walker A and Walker B (52), have been found in the amino acid sequence. Based on the hydropathy profile, the NatB protein is very hydrophobic with six putative transmembrane segments. It shows a modest homology to the hydrophobic regions of some proteins involved in sodium transport. In view of the sequence analysis, we propose that the gene disrupted in the JC901 strain is a part of a two gene ABC type transport or traffic ATPase for Na^+. It is possible that the *natAB* gene products do not function as a Na^+ transporter. They could stimulate other Na^+ transport system(s). Or a mutation in some gene locus other than *natAB*, which may have occurred during the transposition of Tn*917* results in the Na^+ sensitive phenotype of JC901. However, the *natAB* gene, when expressed alone in the *E. coli* EP432 strain, complemented this strain which is deficient in two Na^+/H^+ antiporter encoding genes, *nhaA* and *nhaB*. The gene products of *nat*AB functionally complemented the growth phenotype of the *E. coli* strain and restored its energy-dependent Na^+ extrusion capacity. Upon re-transformation of cloned *natAB* genes into JC901, the gene products also restored its Na^+ resistant phenotype and active Na^+-extrusion (14). The sole product of *natB* does not show that function as examined in *E. coli* (Figure 10 and 11). These data indicate that the NatAB proteins themselves are directly related to Na^+ transport, hence the *nat* designation. The deduced structure of NatA and NatB strongly indicate this transport system is of the ABC type. NatA presumably functions, as its sequence suggests, as an ATPase that energizes transport and NatB functions as the membrane component that provides an essential part of the translocation pathway with its six transmembrane segments. Expression of *natB* alone is toxic to *E. coli* EP432, enhancing Na^+ entry into these sensitive cells. It does not, itself, catalyze Na^+ exclusion.

Transporters of the superfamily of ATP-binding cassette proteins carry out a wide variety of substrates across biological membranes. Some members of this family are involved in extrusion of a variety of toxic agents, such as ions, chemical compounds and peptides (3,46). The nat*AB* genes could encode a member of a small subfamily of bacteria ABC-type transport systems which has been named ABC-2 by Reizer *et al.* (110). The closest sequence similarity to *natA* was exhibited by *drrA* of the daunorubicin and doxorubicin efflux system from *Streptomyces peucetius* (35). Based on the molecular model of the four domain structure of ABC type transports (52), we propose the model for the NatAB transport system in which two homodimers each of NatA and of NatB together form the core structure of four domains (shown in Figure 12). The *natB* product is likely to be very toxic in the presence of Na^+ as it was observed that the expression of *natB* alone in *E. coli* increased the sensitivity of host cells to Na^+. Although the data presented do not firmly establish the translational start site of *natB*, we propose that the *natB* is translated from the downstream start codon of the two possible start sites. The region between *natA* and *natB*, with its extensive predicted secondary structure for RNA, might provide a mechanism for preventing the *natB* from being translated without the translation of *natA*.

Since the Na^+ sensitive phenotype of JC901 was only observed at alkali pH, it was of interest to test if this phenotype is due only to a reduction in Na^+ extrusion capacity or also related to a problem with pH homeostasis. In a pH shift experiment, Guffanti showed the JC901 strain possessed a normal capacity for Na^+ or K^+-dependent pH homeostasis (14). From these data, we conclude that the NatAB system is not involved in pH homeostasis. In the next chapter, we will discuss, another gene of *B. subtilis*, the *tetA*(L) gene whose product is the major component involved in pH homeostasis as well as having a role in Na^+-resistance. The

physiological role of the NatAB system maybe involved in Na^+-resistance in the presence of high concentrations of sodium, which cannot be completely handled by the *tetA*(L) gene product.

The β-galactosidase activity of JC901 strain indicates that the transcription level of *natAB* is relatively low, compared to other genes of *B. subtilis.* Expression of *natAB* can be modestly induced by the presence of NaCl and KCl. K^+ had a consistently greater effect than Na^+, and since choline chloride did not induce, the effect is solely dependent upon the cation rather than the anion. Expression of *natAB* was not induced at elevated pH or high osmolarity. Na^+ did not become a more potent inducer at elevated pH as it was reported for E. coli *nhaA* (66). Data on the gene expression induced by Na^+ support that NatAB system are involved in Na^+ extrusion. Most notably, K^+ also induced the expression of the *natAB* genes, which indicates that *natAB* may also be involved in K^+ transport. In the study of over-expression *natAB* in JC901, Guffanti showed that the over-expression of NatAB system increased K^+ uptake. Two possible models might explain K^+ uptake stimulated by NatAB. First, NatAB itself might catalyze coupled Na^+ extrusion and K^+ uptake. Alternatively, the extrusion of Na^+ by NatAB system might enhance the membrane potential which would function as the driving force for K^+ uptake which is mediated by a secondary K^+ uptake system. To test these models, an experiment was designed to detect the effect of CCCP on K^+ uptake. JC901 cell transformed with pJN1 was loaded with Na^+ . Upon the dilution of the cells into a potassium containing medium, the uptake of K^+ was monitored by using ^{86}Rb as mimic. Since CCCP stimulates the extrusion of Na^+, if K^+ uptake was coupled with the same reaction, the presence of CCCP would increase K^+ uptake. On other hand, if K^+ uptake was mediated by a secondary uptake system which used membrane potential as the energy source, the presence of CCCP would inhibit K^+ uptake. The data obtained from the experiment supported

that electrogenic extrusion of Na^+ by NatAB indirectly increased the K^+ uptake by stimulating (an) electrogenic K^+ transporter(s) (14). In the studies of the expression of the promotorless *lacZ* gene in JC901, in addition to the stimulating effect of ethanol, CCCP also induced the expression of the gene by 3 ~ 4 fold. Based on the expression study, the sensitivity of JC901 to growth inhibition by ethanol and CCCP was tested. When compared with wild type strain, JC901 showed a sensitive phenotype to both ethanol and CCCP and this phenotype was functionally complemented by pJN1. Since ethanol and CCCP disrupt membrane structure, the consequence of the treatment with ethanol or CCCP could be accumulation of cytoplasmic Na^+ and loss of cytoplasmic K^+ upon reduction of the normal proton motive force (Δp). In such circumstance, expression of *natAB* could compensate for some of the compromise of membrane barrier function caused by ethanol or CCCP by restoring Na^+ efflux and secondary K^+ accumulation.

Taken together, we would propose that the *natAB* system is an inducible transporter that allows *B. subtilis* to compensate for a compromise of membrane barrier function caused by agents such as ethanol. Like treatment with CCCP, this compromise would result in a partial dissipation of Δp. Upon such dissipation, cells would experience a loss of cytoplasmic K^+ and accumulation of cytoplasmic Na^+. As a primary transport, NatAB could catalyze efflux of the Na^+ and indirectly stimulate uptake of the K^+. Perhaps alcohol substrates of the putative *orfC* product are some products produced by plants and found in the natural environment of *B. subtilis,* which could function as membrane perturbants. The other function of this operon could be involved in inactivation of these substrates.

A Na^+/H^+ Antiporter Encoded by The Chromosomal *tetA*(L) gene

Results:

1) The transposon insertional site in strain JC111.

The flanking region of the Tn*917* insertion in JC111 was cloned as described under Materials and Methods. The transposition site was found to be in the promotor region of the *tet* locus. This gene had already been sequenced and mapped on the chromosome (94). A diagram of the locus, using data obtained from Genbank™ is shown in Figure 13. The nucleotide sequence of the promotor region is shown in Figure 14.

2) Growth properties and JC111.

The growth properties of JC111 were compared to wild type in both TKM and TTM media. As shown in Figure 15, wild type and JC111 grew equally well at pH 7 in TKM medium in the absence of added NaCl. Although not shown, addition of NaCl was not inhibitory to JC111 at pH 7. At pH 8.3, the wild type grew far less well than at pH 7, and was slightly inhibited by addition of NaCl. The JC111 strain was more inhibited by the elevated pH, and exhibited sensitivity to added NaCl. Both wild type and JC111 strains were also grown in TTM medium in which the only added monovalent cation was 1 mM potassium phosphate. As shown in Figure 16, wild type and JC111 grew well in this medium at pH 7, and both strains grew poorly at pH 8.3. Addition of either NaCl or KCl enhanced the growth, but the Na^+-dependent growth and K^+-

dependent growth of JC111 was not so good as those of the wild type.

3) Energy-dependent Na^+ efflux of JC111.

Whole cells of wild type and JC111 strains were starved briefly and loaded with $^{22}Na^+$ by incubation with 5 mM radiolabelled NaCl at pH 8.5 for about 2 hours. Cells were re-energized by two protocols. The efflux of $^{22}Na^+$ was monitored upon the energization.

In one protocol, energization of the efflux was achieved by the establishment of a valinomycin-mediated potassium diffusion potential. This negative in and positive out potential could energize electrogenic antiport. The control for this experiment was conducted by diluting the cells into medium which contained a high concentration of potassium. The outside potassium prevented the development of a potential. As shown in Figure 17, Na^+ efflux driven by a diffusion potential was observed in both strains, but efflux from JC111 was considerably slower than that from wild type strain. In the second protocol, an electron donor, malate, was added to establish an electrochemical proton gradient which could provide the energy for the efflux mediated by antiport. The efflux from JC111 was significantly slower than that from the wild type stain. The effect of uncoupler CCCP on the efflux was tested, and it significantly inhibited the efflux of $^{22}Na^+$ by both wild type and JC111. (Figure 18).

4). Expression of the cloned *tetA*(L) gene in *E. coli* NM81 strain.

The *tetA*(L) transporter gene was amplified alone from *B. subtilis* chromosomal DNA by PCR, and cloned into pGEM3Zf(+) under the control of the T7 promoter as described under

Materials and Methods. The resulting plasmid, pJTA1, was then transformed into *E. coli* NM81 strain, which carries a deletion in Na^+/H^+ antiporter-encoding gene *nhaA*. Growth of this strain is inhibited by NaCl concentrations above 600 mM NaCl at pH 7.5 because of the deletion. The transformant was tested for its Na^+-resistance and Na^+/H^+ antiport activity. In the Na^+ resistance experiment, the transformant of pJTA1 was grown on LBK containing 600 mM NaCl along with the control transformant with pGM36, which contained the cloned *E. coli* antiporter-encoding gene *nhaA* (positive control), and a transformant with pGEM3Zf(+), which is the host plasmid of pJTA1 (negative control). As shown in Figure 19, upon transformation with pJTA1, *E. coli* NM81 regained the ability to grow on the LBK in the presence of 600 mM NaCl, although the pJTA1 transformant did not grow as well as the pGM36 transformant. The pGEM3Zf(+) transformant, as expected, did not exhibit growth on 600 mM NaCl.

Extensive studies were subsequently conducted by Guffanti on everted vesicles of *E. coli* transformants expressing pJTA1 (34). These studies indicated that the TetA(L) protein catalyzes both Na^+/H^+ and metal-tetracycline/H^+ antiports with a higher affinity for Na^+/H^+ antiport. The optimal metal was cobalt. Na^+/H^+ antiport activity has a lower Km and Vmax at alkaline pH. On the other hand, metal-tetracycline/H^+ antiport exhibited both higher velocity and a lower Km at neutral pH than at alkaline pH. Na^+ does not compete with tetracycline uptake, nor does tetracycline or cobalt or both compete with Na^+ uptake by the everted vesicles.

5) Transcription of the *tetA*(L) gene in JC111.

RNA was prepared from wild type, JC111 and JC111/pCIS7 strains. Northern analysis was carried out by using the *Pst*I probe (R1 in Figure 13). As shown in Figure 20A, two bands

of different sizes were found in each strain. The lower band with size about 1550 nt was the anticipated transcriptional product of the *tetA*(L) gene, based on the locations of putative promoter and a rho-independent terminator sequence which was about 50 nt downstream of the coding sequence (Figure 14). The larger size hybridizing band was predicted to be about 2100 nt which could be transcribed from an upstream promotor or could be a transcriptional read-through product (passing the rho-independent terminator sequence). No apparent difference in either signal intensity or size was found between wild type and JC111, while the JC111/pCIS7 strain exhibited much stronger bands of the same size. Since the transposon Tn*917* was inserted at the putative promoter region, this location made it unlikely that the larger RNA product was transcribed from an upstream promoter. In that case, transcription of this RNA in the JC111 strain should have been altered by the transposon. A second probe which corresponded to the downstream sequence of the *tetA*(L) gene coding region (R2 in Figure 13) was used to probe the same RNA sample. As shown in Figure 20B, with this probe, as expected, the smaller signal band was not detected. The larger band of the same size detected by the R1 probe was found in wild type, JC111 and JC111/pCIS7 strains and exhibited the same pattern as detected by the R1 probe. Apparently, the larger RNA was a transcriptional read-though product. However, further examination of the sequence several hundred nucleotides downstream of the *tetA*(L) gene coding region reveal no ORFs. Thus the significance of the read-through, if any, is currently unclear.

A single transcriptional start has been mapped for the plasmid *tet*(L) gene of pTHT15, but the mRNA start site of *tetA*(L) in *B. subtilis* had not been mapped. Reverse transcriptase mapping of the transcriptional start site was therefore conducted. Two mRNA start sites have been found as shown in Figure 21. One mRNA start site matches the start site mapped in plasmid

tet (L) (pTHT15)(51), and the new start site, with a strong signal, is just five bp upstream from the first start site. There are good candidates for -10 elements for both transcriptional start sites, but only the downstream start site has a good candidate for a -35 sequence.

6) Deletion of the *tetA*(L) gene from the wild type strain.

The finding of *tet* mRNAs in the JC111 strain that was not different from those in the wild type was unexpected, given the phenotype of JC111. It is unlikely that the detection of RNA species was due to cross-hybridization with other RNAs which are not encoded by *tetA*(L) gene, since two probes corresponding to different sequences were used in the Northern analysis and the patterns were found to be the same. Also in the JC111/pCIS7 strain which carries multicopies of the *tetA*(L) gene, the size of the detected signal was the same as that in wild type and JC111, but with enhanced intensity. The possible explanation for the Na^+ sensitive phenotype of JC111 is that the insertion of the transposon may alter the regulation of the *tetA*(L) gene expression. To address this question, a *B. subtilis* strain was constructed in which the *tetA*(L) gene was replaced with a chloramphenicol acetyltransferase (*cat*) marker gene, described under Materials and Methods. Northern analysis of RNA isolated from the deletion strain, designated JC112, showed that two bands identified in wild type, JC111 and JC111/pCIS7 disappeared, which confirmed that those RNAs were the products of the *tetA*(L) gene (Figure 20). Southern analyses of the chromosomal DNA isolated from JC112, using probes of both the *tetA*(L) and the *cat* gene coding regions also indicated that the *tetA*(L) gene was replaced by *cat* gene (Figure 22).

All deletion strains were selected for chloramphenicol resistance after transformation with plasmid pJBC3 in which the *tetA*(L) gene was replaced with the *cat* gene. Six of the

chloramphenicol resistant colonies were further characterized. These strains were found to possess phenotypes of various intensities with respect to both Na^+ sensitivity and pH homeostasis, but there was a parallel deficiency in two properties. The phenotypes ranged from a nearly wild type phenotype down to the severely compromised phenotype of JC112 (described further below). Southern analysis of all six strains indicated that the *tet* locus had been deleted (*data not shown*). The range of different stable phenotypes of the deletion strains strongly suggested that secondary mutations were present and perhaps required for the strains to survive upon the deletion of the *tetA*(L) gene. It had been reported earlier that the *tetA*(L) gene might be vital for the wild type strain after attempts to delete it were unsuccessful (56). Most likely, JC112, which presented the strongest phenotype, also contains a second site mutation, but it was the most minimal compensatory change allowing a stable viable deletion of *tet*. Subsequent evidence was obtained for a regulatory mutation in JC112 that may up-regulate a modestly compensatory, alternate antiporter.

The phenotype of JC112 was investigated in order to compare its severity to that of the JC111 and wild type strains. As shown in Figure 15, JC112 exhibited a much more severe phenotype than that of JC111. It showed a greater sensitivity to both Na^+ and elevated pH (Figure 15). Both Na^+ and K^+-dependent pH homeostasis was also impaired. As shown in Figure 16, growth of JC112 was very poor in TTM medium At pH 7.0, even without substantial concentrations of monovalent cations, while growth of JC111 was similar to that of wild type. At pH 8.3, in the absence of monovalent cations, the growth of JC112 was almost totally inhibited. Upon the addition of either NaCl or KCl, the growth of JC112 at pH 8.3 was increased just slightly and it was not nearly as good as that of JC111 which was also impaired

relative to the wild type. Notably, the JC112 strain also showed greater tetracycline sensitivity than the wild type when examined at low antibiotic concentrations (16).

7) Overexpression of a his-tagged *tetA*(L) gene product.

The *tetA*(L) gene was cloned into an expression vector with hexahistidine tag added to the C-terminus. With the addition of hexahistidine, the gene product could be conveniently purified by Ni-resin (Qiagen). The expression of the *tetA*(L) gene was controlled by the *lac* operator. *E. coli* DH5α transformed with two plasmids pJQ2 and pREP4, was grown in LB + 2% glucose. 5 ml of overnight culture was inoculated into 1 liter LB + 2% glucose. After the cells grew to $A_{600} \doteq 0.7$, Cells were harvested by centrifugation and resuspended in 1 liter fresh LB containing 2 mM IPTG. With another 2 hours of growth, cells were harvested and membranes were isolated. The protein was purified as described under Materials and Methods. As shown in Figure 23, upon the addition of IPTG, a new protein band was found in the membrane samples after initial Ni-resin and EDTA purification. The size of the product was calculated about 34 kDa on a single 10% SDS- polyacrylamide gel or 38 kDa on a 10 to 20 % gradient SDS-polyacrylamide gel, much smaller than the calculated 49 kDa. It has been observed that most hydrophobic proteins run smaller than their actual size on SDS-polyacrylamide gel. This protein was recovered, and the N-terminus was sequenced in the Protein Chemistry Core Laboratory of Mount Sinai School of Medicine. The first six N-terminal amino acids of the purified protein are SQSTLR, which represent amino acids # 10 - 15 of the deduced N-terminal sequence. Four amino acids from the vector and five from the *tetA*(L) gene were missing. The loss of the N-terminal amino acids could be due to the expression of the protein in a heterogenous system or

the natural post-translational processing of the protein. Since no report on the purification of the intact TetA(L) protein has been published, it is hard to distinguish between these two possibilities at this moment. It will be of interest to study the product produced in *B. subtilis* in future.

Western immunoblot analysis was conducted on the same protein samples. 5 μg of the protein samples were run on a 10% SDS-polyacrylamide gel and transferred onto a nitrocellular membrane. The antibody used in the experiment was prepared against the N-terminal of the Tet protein (described under Material and Methods). As shown in Figure 24, in the IPTG-induced protein sample, a strong band was detected of the same size as the Tet band seen in Figure 23. A faint band was found in the uninduced sample, which suggested that, even without induction, the promoter for this expression system was leaky.

8) Purification and reconstitution of the his-tagged TetA(L) protein.

Rigorous demonstration of that the TetA(L) protein functions as both Na^+/H^+ antiporter and tetracycline/H^+ antiporter requires that the purified and reconstituted protein exhibit both activities. The his-tagged Tet protein was purified from the host *E. coli* strain. After induction by IPTG, cells were broken by French pressure and the membrane fraction was collected. The Tet protein was released from the membrane and purified via Ni-NTA resin as described in Materials & Methods. The purified Tet protein was analyzed on an SDS-polyacrylamide gel. As shown in Figure 25, a major Tet protein band was observed. The purified protein was then reconstituted into proteoliposomes. The Na^+/H^+ and tetracycline/H^+ antiport activities of the Tet protein were assayed by ΔpH-driven ^{22}NaCl uptake and [^{3}H]tetracycline uptake in the presence of cobalt. When Tet protein containing proteoliposomes were diluted into choline buffer, a pH gradient was

imposed (acid inside). As shown in Figure 26 and 27, both Na^+ and tetracycline uptake was driven by this pH gradient, proving direct evidence that the TetA(L) protein is a multifunctional transporter which catalyzes both Na^+/H^+ and tetracycline/H^+ antiport.

Discussion:

Based on the sequence data and prior reports (54,94,117,118,123), the chromosomal *tet*A(L) gene locus of *B. subtilis* encodes a tetracycline efflux antiporter. It exists as a single copy near the origin of replication and all strains which contain this gene are sensitive to the usual challenge concentration of tetracycline. Upon amplification of the gene locus, the strain gains tetracycline resistance. Our data suggest that the same gene product functions as an active, electrogenic $Na^+(K^+)/H^+$ antiporter which plays a significant physiological role in Na^+-resistance and in Na^+- and K^+-dependent pH homeostasis at alkaline pH. This is the first suggestion that a Tet protein is involved in Na^+ efflux and plays physiological roles in pH homeostasis. Some other Tet proteins have been reported to catalyze K^+ transport (24,30,31,33,91), but none of them has been shown to have physiological roles unrelated to tetracycline resistance.

Insertion of the transposon in the promoter region results in a phenotype of JC111. When JC111 was grown at elevated pH (8.3), it showed sensitivity to both Na^+ and high pH. When the wild type strain was grown at pH 8.3, it needed either K^+ or Na^+ to exhibit optimal growth. JC111 partially loses the ability to use theses cations, especially Na^+, in support of growth at elevated pH. The energy-dependent Na^+-extrusion of JC111 is also impaired. A decrease in diffusion potential-dependent Na^+ efflux indicates that Na^+/H^+ antiport by TetA(L) is electrogenic and

proceeds with a H^+:Na^+ ratio greater than one. The cloned *tetA*(L) gene was also expressed in a Na^+/H^+ antiporter deficient *E. coli* strain (NM81) (96). The gene product complements the Na^+ sensitive phenotype of this strain.

In a Northern analysis of RNA isolated from JC111, a wild type transcription pattern of the *tetA*(L) gene was observed. This unexpected finding raised the question that, if the *tetA*(L) gene is normally transcribed as wild type, what accounts for the phenotype of JC111? One possibility is that the insertion of the transposon caused one or more mutations in the *tetA*(L) gene which resulted in a mutant Tet protein without function. However, we cloned and sequenced the whole coding region of the *tetA*(L) gene. No mutation was found. Another possibility is that the insertion of the transposon altered the regulation of the *tetA*(L) gene expression. If that is true, we expected that a *tetA*(L) deletion strain might exhibit an even more severe phenotype than JC111 does. So JC112 was constructed in which the whole *tetA*(L) gene locus was replaced by the *cat* gene. Indeed, JC112 shows a stronger phenotype than JC111 especially with respect to growth at pH 8.3. Also in contrast to JC111, the phenotype of JC112 could also be observed even at pH 7.0. Another phenomenon was also observed during the isolation of JC112. Among all the deletion strains, different phenotypes were found with respect to both Na^+ sensitivity and pH homeostasis, which range from a nearly wild type-like phenotype to the most severe phenotype of JC112. TetA(L) is the dominant antiporter involved in Na^+-resistance and in both Na^+- and K^+-dependent pH homeostasis. Neither Na^+- nor K^+-dependent pH homeostasis was detected in JC112 (16). Perhaps deletion of the *tetA*(L) gene is lethal to the host strain. Unless compensated for by one of several secondary mutations, the host would not survive in the medium even at pH 7.0. The different secondary mutations compensate to different degrees which results in the

different phenotypes. Most likely, JC112 contains such a mutation, but it is the least compensatory change. Ives and Bott (56) reported that they failed in several attempts to delete the chromosomal *tetA*(L) gene. They concluded that the gene was vital to the host cells. The possible reason for their failure to delete the gene may be that they did not eliminate the Na^+ from the medium, since Na^+ is normally included in most media.

In the growth experiments, at elevated pH, the wild type strain needed either Na^+ or K^+ to exhibit optimal growth. This suggests that neutralophilic *B. subtilis* can use both Na^+ and K^+ for pH homeostasis while Na^+ is more efficient than K^+. *B. subtilis* is different from alkaliphilic prokaryotes which specifically require Na^+ for pH homeostasis (15,72). In JC111 and JC112, the loss of K^+-dependent pH homeostasis indicates that, in addition to catalyzing Na^+/H^+ antiport, the *tetA*(L) gene product also catalyzes K^+/H^+ antiport. Upon the transformation of cloned *tetA*(L) gene into the JC112 strain, the transformant showed a phenotype almost same as wild type. Growth data are very consistent with the data from a pH shift experiment conducted by Guffanti (16), in which the cells growing at pH 7.5 were quickly shifted to the media at pH 8.5 with different additions. After 10 minutes, the cytoplasmic pH was measured. JC112 completely loses the capacity for both Na^+-dependent and K^+-dependent acidification of the cytoplasm, which strongly suggests that the *tetA*(L) gene product plays a major role in both Na^+- and K^+-dependent pH homeostasis. Again, transformation of JC112 with a cloned *tetA*(L) gene restored the Na^+ and K^+ dependent pH homeostasis and normal growth resistance to Na^+ (as shown in Figure 15 and 16).

In an attempt to demonstrate rigorously that the *tetA*(L) gene product functions as both Na^+/H^+ and tetracycline/H^+ antiporter, a his-tagged Tet protein was overexpressed and purified

in *E. coli*. The purified Tet protein was then actively reconstituted into proteoliposomes. Upon a pH gradient was imposed across the membrane that serves as the energy source of the transport, the uptake of both Na^+ and tetracycline was observed. Although a Gram-negative Tet protein (TetA(B)) has been purified (1,45), the only reconstitution reported involved a low activity of tetracycline-metal/H^+ antiport in a mixed reconstitution system with the F_1F_0 ATPase (128). There is still much optimization of both the purification and reconstitution to be done, and a detailed set of experiments in driving force, kinetics, substrate range and inhibitors. Nonetheless the data we present here are the first example of a purified and functionally reconstituted Tet protein in proteoliposomes and they are also the first direct evidence which indicates that Tet protein is involved in a physiological role other than tetracycline resistance.

Based on their inducibility of plasmid- and transposon-borne *tet* genes by tetracycline (55,88), the *tet* genes of the efflux type from Gram-positive bacteria have been predicted to be regulated in their expression at the level of translation attenuation (122), by analogy with other antibiotic resistance loci (25,80). The regulation of the *tetA*(L) gene has also been investigated in our laboratory (16). A *tetA*(L)-*lacZ* fusion gene was integrated into the *amy*E locus of both the wild type and JC112 strains, so that the expression of the fusion protein, which is transcribed from the *tetA*(L) promoter, could be monitored by assaying β-galactosidase activity. Monovalent cations, elevated pH and especially tetracycline increased the expression of the fusion gene in the wild type. Some assays were also performed with the JC111 strain, in which, the *lacZ* gene of the transposon in under the control of the half *tet* promoter (only -35, no -10). Although the basal level activity of the JC111 strain is much higher than that of the wild type in which the fusion gene was introduced, the induction of the gene expression by Na^+, K^+ and elevated pH is still

detectable, but the effect of tetracycline is not observed. This suggests that the monovalent cation and alkaline pH-mediated regulation likely rely on regions upstream of the transposon insertional site or of the region between two promoter elements. In contrast, the tetracycline-mediated regulation depends upon elements that are downstream of the insertion. It is possible that there are even more promoter elements or other activating elements further upstream than I have studied. The Na^+ sensitive phenotype of JC111 could be due to the disruption of the up-regulation of the *tetA(L)* gene mediated by monovalent cation and alkaline pH which is dependent on the region upstream of the insertional site of the transponson. This up-regulation no longer occurs in JC111 strain. Another interesting phenomenon is that the basal level of the expression of the *tetA*(L)-*lacZ* fusion in JC112 strain is about 10 fold higher than in wild type strain. This is consistent with the hypothesis that a secondary mutation exists in JC112 strain that could up-regulate the expression of a compensatory antiporter. Additionally, a transcriptional read-through product was found in Northern analysis and the role of this product is still to be clarified.

Table I. Plasmids used in this study.

Plasmid	Properties	Source
pGEM3Zf(+)	*E. coli* cloning vector, Amp^R	Promega
pGEM9Zf(-)	*E. coli* cloning vector, Amp^R	Promega
pSE420	*E. coli* cloning vector, Amp^R, $lacI_q$	Invitrogen
pQE12	*E. coli* expression vector, Amp^R	Qiagen
pREP4	*E. coli* plasmid, Km^R, $lacI_q$	Qiagen
pGM36	cloned *nhaA* in pBR322, Amp^R	E. Padan
pJN1	cloned *natAB* in pGEM3Zf(+), Amp^R	this study
pJN2	cloned large version of *natB* in pGEM3Zf(+), Amp^R	this study
pJN3	cloned small version of *nat*B in pGEM3Zf(+), Amp^R	this study
pJTA1	cloned *tetA*(L) in pGEM3Zf(+), Amp^R	this study
pJQ2	cloned *tetA*(L) with his-tag in pQE12, Amp^R,	this study
pCIS7	cloned chromosomal *tetA*(L) locus (11.5 kb), Amp^R	K. Bott
pSDC2	cloned pC194 *cat* gene in a pBR322 derivative, Amp^R	D. Bechhofer
pJBC1	cloned *tetA*(L) locus in pGEM3Zf(+), Amp^R	this study
pJBC2	cloned pC194 *cat* gene in pSE420, Amp^R	this study
pJBC3	replacement of *tetA*(L) gene with pC194 *cat* gene, Amp^R	this study
pYH56	Shuttle vector, pUB110-pGEM joint replicon Amp^R, Km^R, Erm^R,	D.Bechhofer
pBK36	Shuttle vector, pUB110-pBR322 joint replicon, Amp^R, Km^R,	K. Zen
pJY1	cloned *natAB* in pYH56 Amp^R, Km^R, Erm^R	this study
pTL1	cloned *tetA*(L) in pBK36, Amp^R, Km^R,	this study

Table II. Bacterial strains used in this study.

Bacterial strains	properties	source
Bacillus subtilis:		
BD99, wild type	*hisA1 thr-5 trpC*	A. Garro
JC901	BD99 with Tn*917* insertion in *natB*	this study
JC111	BD99 with Tn*917* insertion in *tetA*(L) promotor	this study
JC112	BD99 with *tetA*(L) deletion	this study
Escherichia coli:		
EP432	K12 with *nhaA* and *nhaB* deletions	E. Padan
NM81	K12 with *nhaA* deletion	E. Padan
DH5αMCR	F', *mcrA, (mrr-hsd, RMS-mcrBC), φ80dlacZΔM15,* Δ(lacZYA-argF)U169, *deoR, recA1, endA1, supE44,* λ-, *thi-1, gyrA96, relA1*	GIBCO-BRL

Table III. The change in the internal Na^+ concentration of *E. coli* EP432 transformants with pGM36 (*nhaA*) or pJN1 (*natAB*) after treatment with CCCP.

EP432 transformed with pGM36 or pJN1 were grown in LBK/350 mM NaCl and 1% glucose. After cells were grown to $A_{600} \approx 0.7$, the culture was divided into two sets. To one set, CCCP was added to a final concentration of 50 µM. After both sets was incubated at 37°C with shaking for 5 minutes, six 1 ml samples were filtered and washed. Radioactivity was determined by liquid scintillation counting. Six individual determinations were averaged.

Average $^{22}Na^+$, cpm, per 1 ml Sample

^{22}Na+, cpm	EP432/pGM36	EP432/pJN1
Experiment 1: no add	4042	3929
50µM CCCP, 5'	4766	3932
Experiment 2: no add	2931	3295
50µM CCCP, 5'	3407	3267
Experiment 3: no add	2651	6030
50µM CCCP, 5'	3060	5545
Experiment 4: no add	3270	5137
50µM CCCP, 5'	3731	5094
%[a] of average increase in cytoplasmic Na+ after CCCP treament	15.2±0.8%	-2.4±3.7%

[a] values shown ±standard deviation of % increase

Table IV. β-galactosidase activity of JC901 cells grown in the presence of different additions.

Cells of JC901 were grown in TTM medium pH 7.0, except where indicated specifically, in the presence of indicated additions for 15 hours. The results shown with standard deviations are the averages of at least six separate determinations.

addition	β-galactosidase activity (μmol/min/ mg cell protein)
None	76 ± 7
None (pH 8.3)	75 ± 12
NaCl, 100 mM	118 ± 10
NaCl, 200 mM	120 ± 15
NaCl, 100 mM (pH 8.3)	144 ± 17
KCl, 100 mM	188 ± 19
KCl, 200 mM	213 ± 22
Choline Cl, 100 mM	61 ± 8
Sucrose, 400 mM	24 ± 9
0.5% Ethanol	174 ± 15
2% Ethanol	222 ± 18

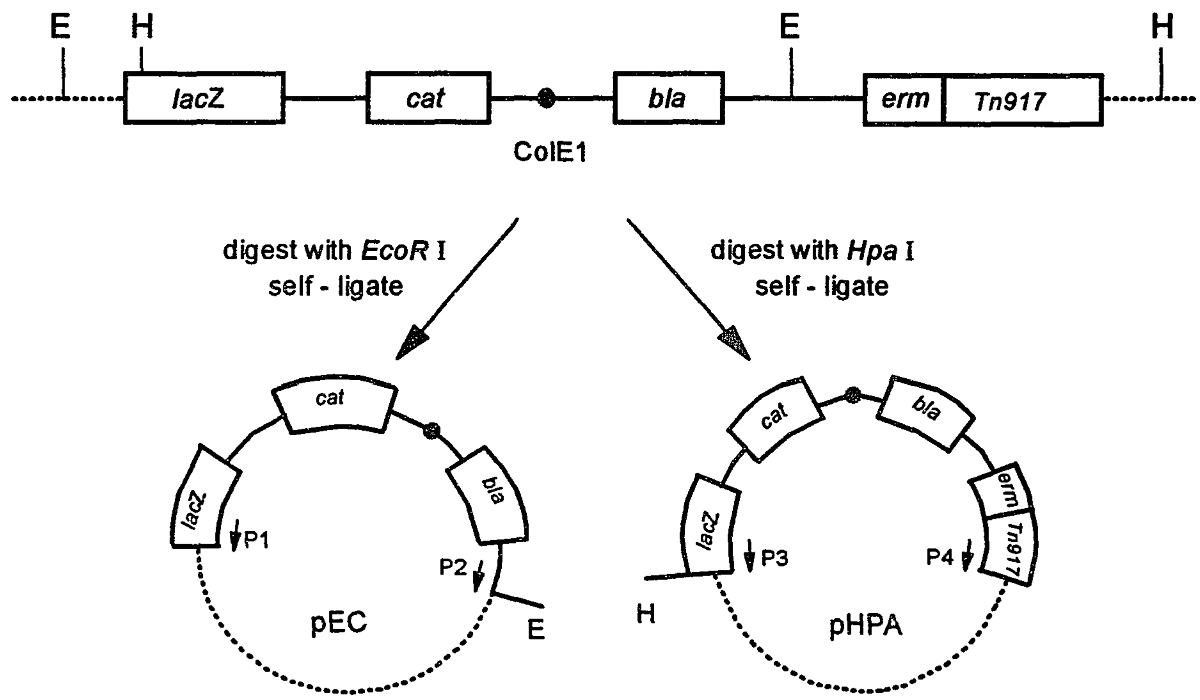
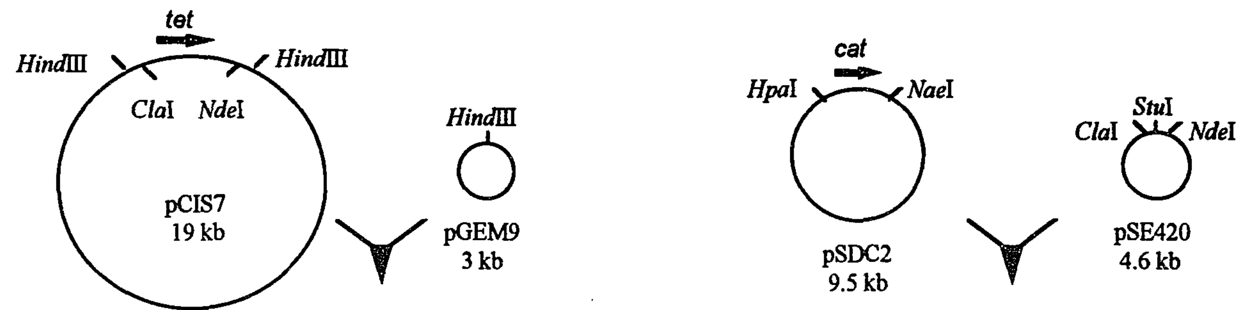


Figure 1. Strategy for cloning the flanking regions of Tn*917* insertional sites. Chromosomal DNA (dotted lines) from both JC901 and JC111 strains was digested separately with *EcoR*I and *Hpa*I, then self-ligated and transformed into *E. coli* DH5α. Three plasmids were subsequently isolated, pEC901, pHPA901 and pHPA111, from, respectively, JC901 with *EcoR*I digestion, JC901 with *Hpa*I digestion and JC111 with *Hpa*I digestion. The locations of four sequencing primers are indicated by arrows. E and H represent, respectively *EcoR*I and *Hpa*I restriction enzyme sites.



isolate the *Hind*III fragment of pCIS7 containing *tetB*(L) and surrounding chromosomal DNA, and insert it into pGEM9

isolate the *Hpa*I and *Nae*I fragment of pSDC2 and insert it into *Stu*I site of pSE420

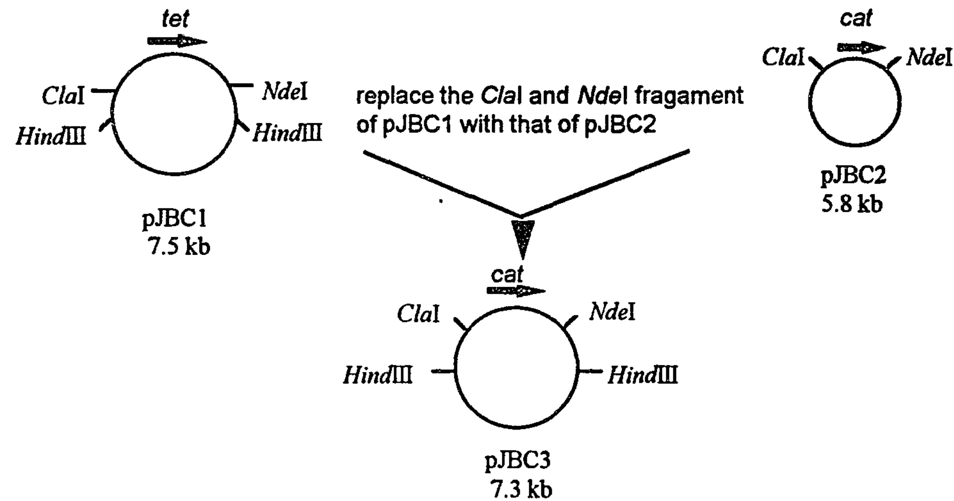


Figure 2. Strategy of making pJBC3 the construct for the deletion of the *tetA(L)* gene from wild type BD99. Two arrows indicate the *tetA*(L) gene and *cat* gene. pSDC2 was obtained from Dr. David Bechhofer and pSE420 was obtained from Dr. Jurgen Brosius.

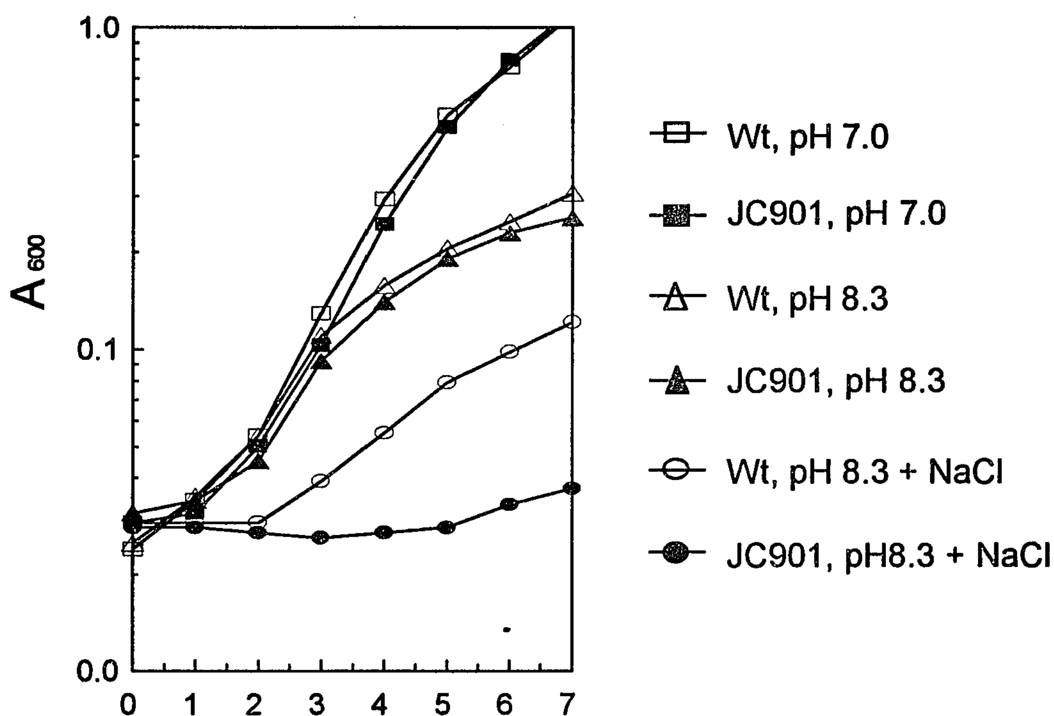


Figure 3. Growth inhibition of the JC901 strain by Na+. Cells of wild type and JC901 are grown in the absence of added NaCl at pH 7.0 or pH 8.3 in SSM. Each strain is also grown at pH 8.3 in the same medium except that NaCl is added to a final concentration of 0.7 M. 0.5 ml of overnight culture (in SSM pH 7) is inoculated into 50 ml of fresh medium. The growth of cells is monitored by recording A_{600} at each hour.

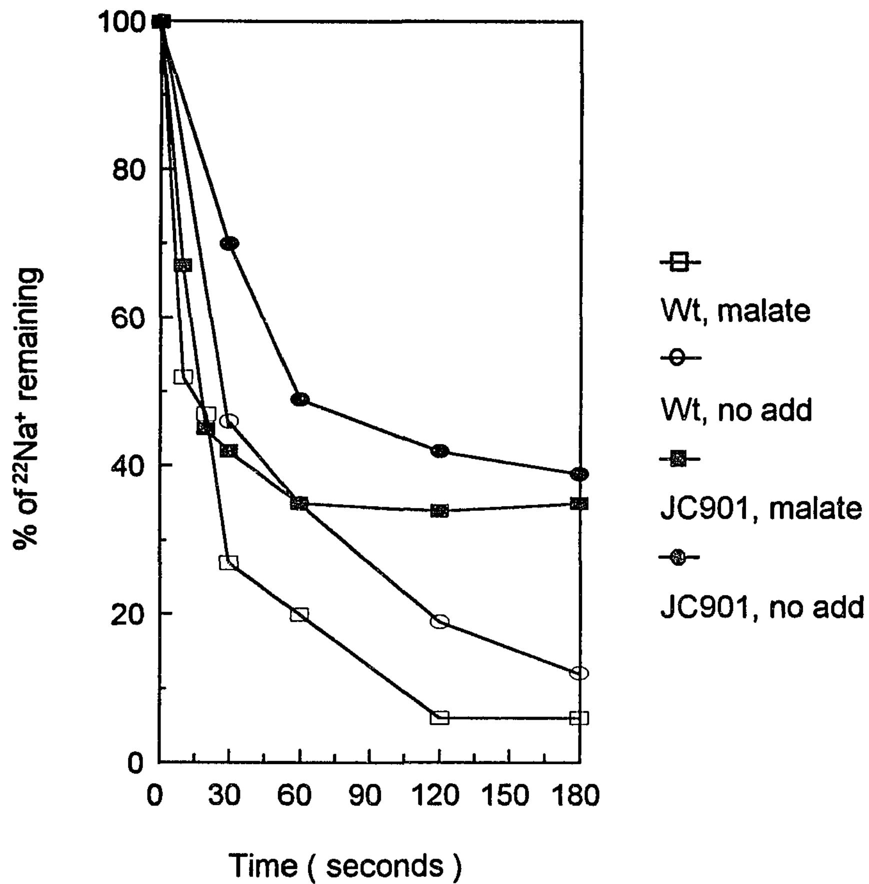


Figure 4. $^{22}Na^+$ efflux from wild type and JC901 cells. Cells of the wild type and JC901 were starved and loaded with $^{22}Na^+$. At zero time, the cells were diluted into 100 mM potassium phosphate buffer, pH 8.5, in the presence and absence of 10 mM malate. Samples were filtered at intervals, and the $^{22}Na^+$ remaining in the cells was determined by scintillation counting.

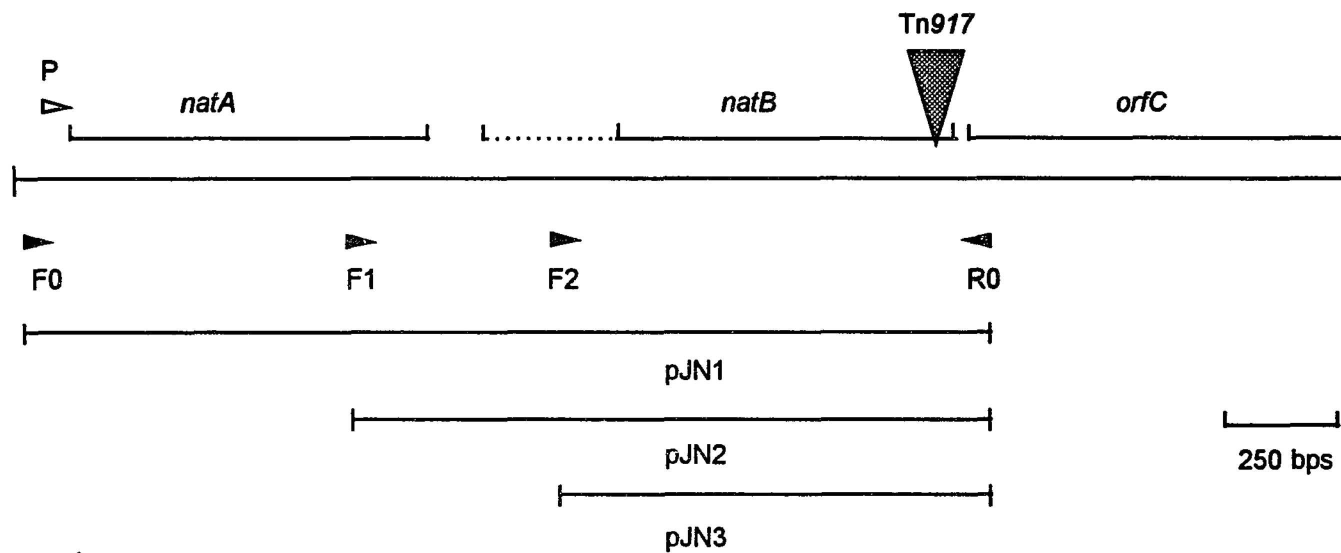


Figure 5. Schematic diagram of the Tn*917* insertional site in JC901. The putative promoter for three ORFs (*orf*C is only partially sequenced) is indicated with theopen arrowhead and two possible translational start sites for natB are indicated, the larger version extending from the smaller by the dotted line. The positions for the primers used in PCR cloning are showed by solid arrowheads and the PCR products cloned into different construts are indicated. The large triangle represents the transposon which inserted at the 3' end of *natB*.

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AATCATCTTTATTCTATCCCATTTCACGACATAAAAAGAACTCTTCGCGACAACTTTATCCAACTAATCCAGCTTTTCGTATATAGTCAT   90
                                                         -35                     -10
TACTAGAAATAAAGATAAGGGAGATTGTGACATGATTACACTGACCGATTGCAGCCGCAGGTTTCAGGATAAGAAAAAAGTAGTCAAAGC  180
                                 M  I  T  L  T  D  C  S  R  R  F  Q  D  K  K  K  V  V  K  A
GGTGCGAGATGTAAGCTTAACAATTGAAAAAGGAGAAGTCGTCGGCATTCTCGGAGAAAACGGTGCCGGCAAAACGACGATGCTGAGAAT  270
  V  R  D  V  S  L  T  I  E  K  G  E  V  V  G  I  L  G  E  N  G  A  G  K  T  T  M  L  R  M
GATTGCTTCCTTGCTTGAACCATCACAGGGTGTAATCACAGTAGACGGCTTTGACACGGTCAAGCAGCCGGCTGAGGTCAAACAAAGAAT  360
  I  A  S  L  L  E  P  S  Q  G  V  I  T  V  D  G  F  D  T  V  K  Q  P  A  E  V  K  Q  R  I
CGGTGTCTTATTCGGAGGAGAAACCGGGCTTTACGACAGGATGACCGCTAAAGAAAATCTCCAATACTTCGGCAGGCTGTATGGGCTGAA  450
  G  V  L  F  G  G  E  T  G  L  Y  D  R  M  T  A  K  E  N  L  Q  Y  F  G  R  L  Y  G  L  N
CCGCCACGAGATCAAAGCAAGAATAGAAGATTTATCGAAACGGTTCGGCATGCGCGATTATATGAACCGGAGAGTGGGCGGTTTTCGAA  540
  R  H  E  I  K  A  R  I  E  D  L  S  K  R  F  G  M  R  D  Y  M  N  R  R  V  G  G  F  S  K
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TGAGGATTTGAATTACATCTTTATGTCTAAGCTTGTCAGGGGGATTTCTTAAATGCTGAGCCATATTTATAAAAAAGAAATGATCGACGC  900
  E  D  L  N  Y  I  F  M  S  K  L  V  R  G  I  S  *
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                                                                                         M
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Figure 6. Nucleotide sequence and deduced amino acid sequences of the flanking region in the JC901strain. The putative promoter for three ORF is indicated (-35, -10). The putative SD sequencesare double underlined and the possible translational start sites are indicated with bold letters. Six putative membrane-spanning segments of *natB* gene product prodicted by hydropathy analysis are underlined. The insertional site of the transposon is indicated by an arrow. Whole sequence is deposited in Genbank™/EMBL with accession number U30873.

Walker A Motif

GXXGXGK

```
NatA (26) LTIEKGEVVG ILGENGAGKT TMLRMIASLL EPSQGVITVD GFDTVKQPAE VKQRIGVLFG GETGLYDRMT
DrrA (28) LNVPAGLVYG ILGPNGAGKS TTIRMLATLL RPDGGTARVF GHDVTSEPDT VRRRISVT.G QYASVDEGLT
ABP   (1) .......... MLGANGAGKT TTFRMMLGLL EPTEGEMTWK GEKI....DY PRTNVIGYLP EERGLYPKLK
```

Loop 3 | Walker B Motif

SXG | hhhhDEP

```
NatA  AKENLQYFGR LYGLNRHEIK ARIEDLSKRF GMRDYMNRRV GGFSKGMRQK VAIARALIHD PDIILFDEPT
DrrA  GTENLVMMGR LQGYSWARAR ERAAELIDGF GLGDARDRLL KTYSGGMRRR LDIAASIVVT PDLLFLDEPT
ABP   VRDQLIYLGR LKGMHKKDII PEMRMWLERF KVTDYETKRI EELSKGNQQK IQFIASVIHR PELLILDEPF
```

```
NatA  TGLDITSSNI FREFIQQLKR EQKTILFSSH IMEEVQALCD SVIMIHSGEV IYRGALESLY ESERSEDLNY (235)
DrrA  TGLDPRSRNQ VWDIVRALVD AGTTVLLTTQ YLDEADQLAD RIAVIDHGRV IAEGTTGELK SSLGSNVLRL (237)
ABP   SGLDPVNAEL LKEAVIDLKK KGTTIVFSSH RMDHVEELCQ HLCILRHGTP VVKGELREIK RSFRNKFI.. (194)
```

Figure 7. A lignment of NatA with putative ATP binding proteins from prokaryotic ABC-type transporters. Two amino acid sequences, DrrA of Streptomyces peucetius (assesion number S27707) and an ATP binding protein from *Bacillus firmus* OF4 (accession nimber S15486) were compared with NatA by using PILEUP program. The conserved regions for ATP binding proteins of ABC type transporters are showed with bold letters.

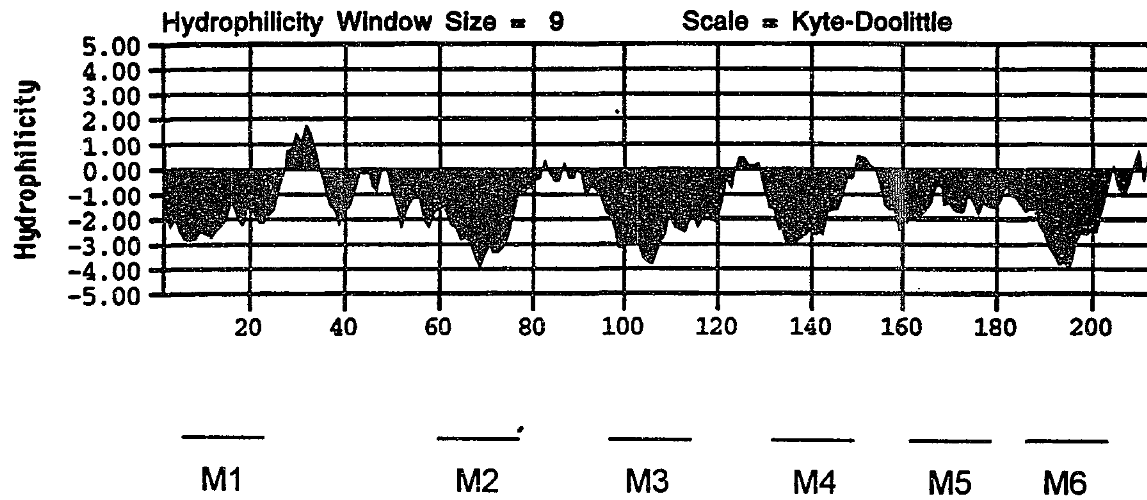


Figure 8. The plot of the hydrophilicity profile of *natB* gene product. The whole coding region is prodicted to be 213 amino acids (smaller version). The hydrophilicity profile is obtained via the Mac-vector program running at window size of nine. The six possible membrane-spanning segments are also indicated.

```
NatB (74) TVLFTENLKT AFQLGDHMWS VIGASALIIV LSALLISAME LFISIMSSSV KEAQSYMSLV
Nhe3 (60) WVLVASLAKI VFHLSHKVTS VVPESALLIV LGLVL..... ......GGIV LAADHIASFT

          VFLPVFPMFF IFSKAPNQFD LSYFLIPFLN LHALFKQLLF GMVDPATILS TSGTIAVLIA IFF (166)
          LTPTVFFFYL L...PPIVLD AGYFMPNRLF FSNLGSILLY AVV..GTVWN AATTGLSLYG VFL (197)
```

Figure 9. Sequence similarity between NatB and rabbit NHE3 (accession number A40205). Identical residues are boxed. The dotted regions indicate gaps generated by the PILEUP program.

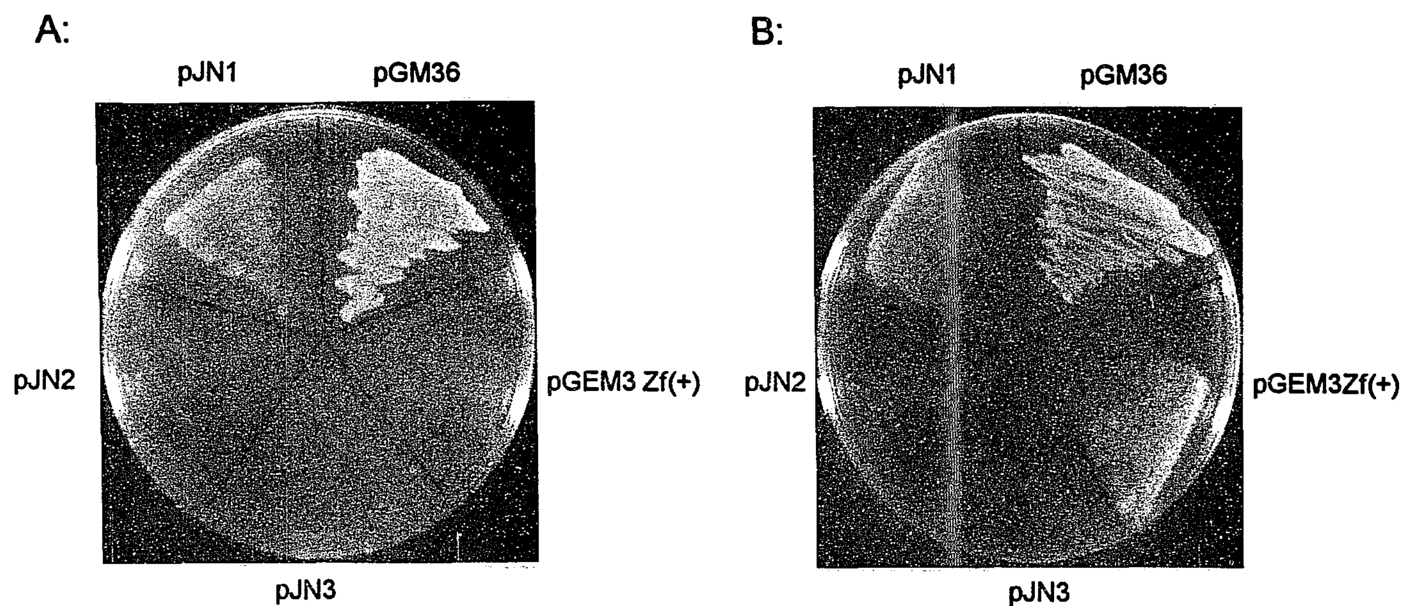
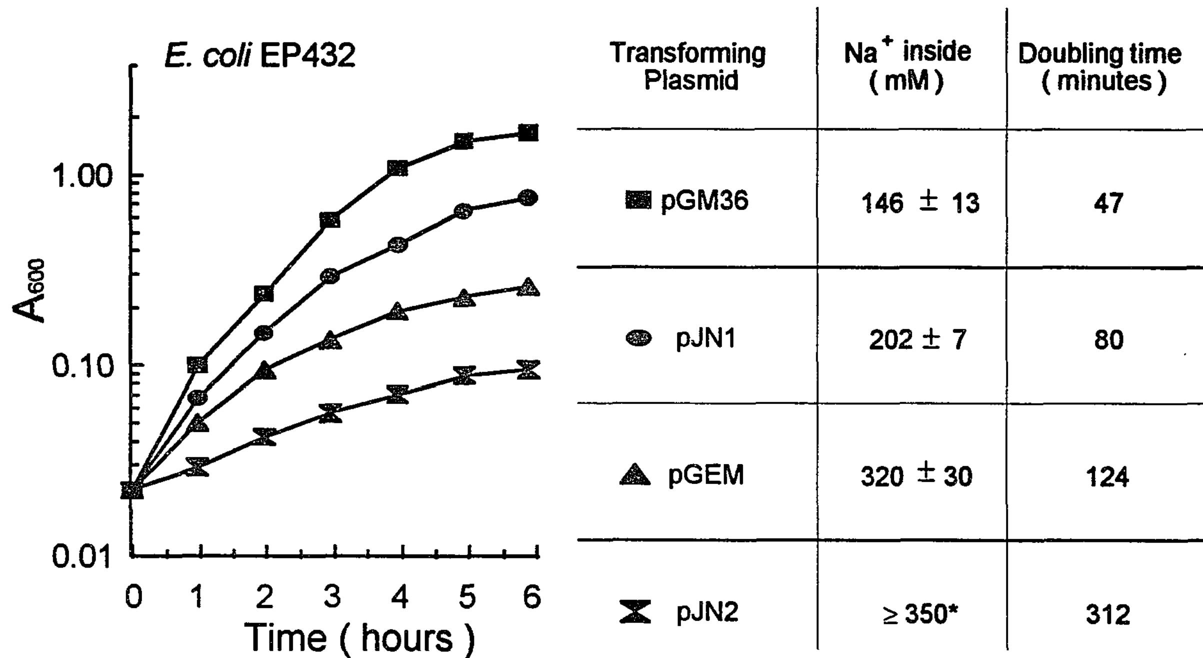


Figure 10. Growth of *E. coli* EP432 transformants on LBK in the presence of NaCl. *E. coli* EP432 transformed by pGM36, pGEM3Zf(+), pJN1 (*natAB*), and the two plasmids expected to express *natB* (pJN2 and pJN3) were streaked on LBK medium containing either 0.4 M NaCl (A) or 0.2 M NaCl (B) and grown at 37°C overnight. Although not shown here, all five transformants grew equally well on LBK in the absence of NaCl.



Transforming Plasmid	Na^+ inside (mM)	Doubling time (minutes)
■ pGM36	146 ± 13	47
● pJN1	202 ± 7	80
▲ pGEM	320 ± 30	124
⧗ pJN2	≥ 350*	312

Figure 11. Na+ sensitivity and exclusion capacity of *E. coli* EP432 expressing *natAB* or *natB*. EP432 transformed with pGM36, pGEM3zf(+), pJN1 and pJN2 were grown in LBK containing 0.35 M $^{22}NaCl$. Growth of each transformant was monitored by measurements of A_{600}. The internal Na^+ concentrations were determined as described under Materials & Methods. The results presented are the averages of duplicate measurements from two independent experiments. *In several experiments, the EP432/pJN2 transformant grew too poorly to perform the measurement. Data shown for this transformant are the results of only one experiment in which it grew better than in other experiments, thus yielding enough cell material for the measurements.

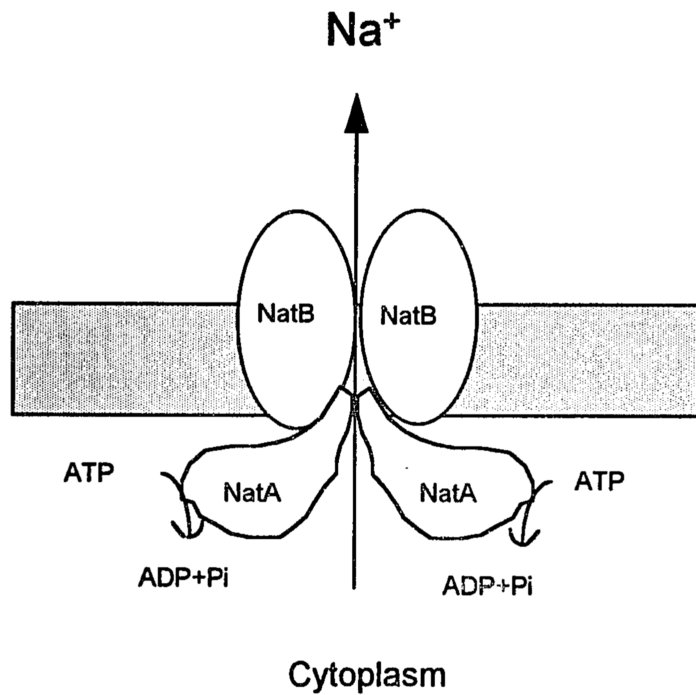


Figure 12. Model for the NatAB transport system. The cytoplasmic membrane is showed by the stippled bar and the arrow indicates the movement of Na^+. The dimer of NatB is incorporated in the membrane and the dimer which hydrolyzes ATP is located largely at the cytoplasmic face of the membrane.

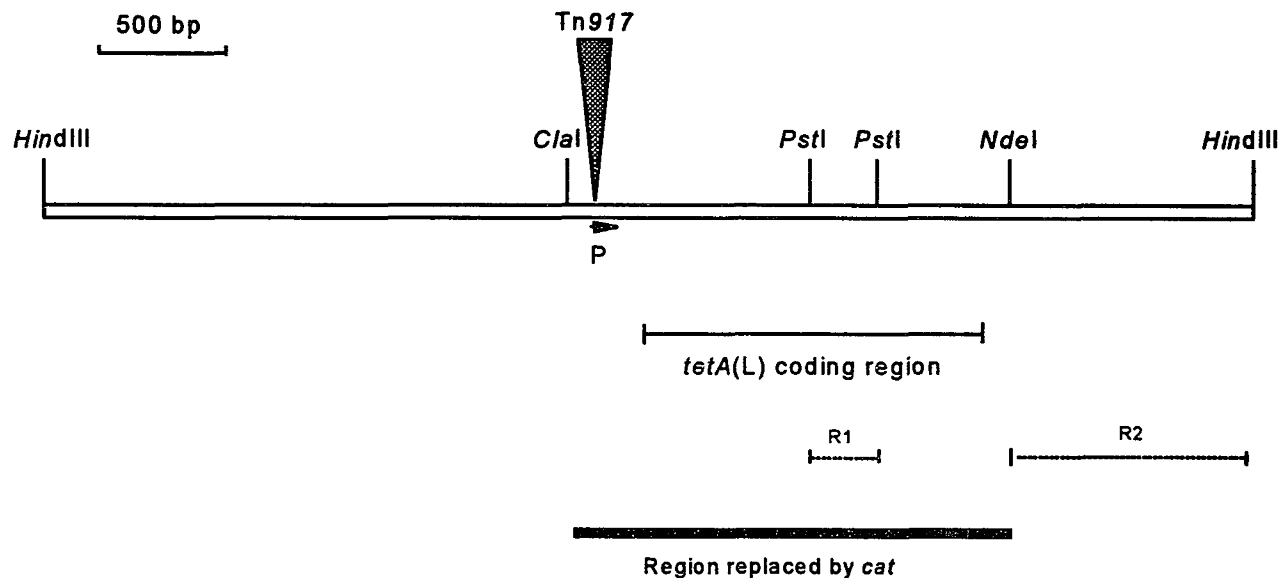


Figure 13. Diagram of the *tetA*(L) region of the *B. subtilis* chromosome. The relevant restriction sites and the *tetA(L)* promoter (P) are indicated. The large triangle indicates the insertional site of the transposon Tn*917* in JC111. The DNA fragment which was replaced by cat in JC112 is also shown (see later). R1 and R2 represent the DNA templates which were used for transcription of riboprobes in Northern analysis.

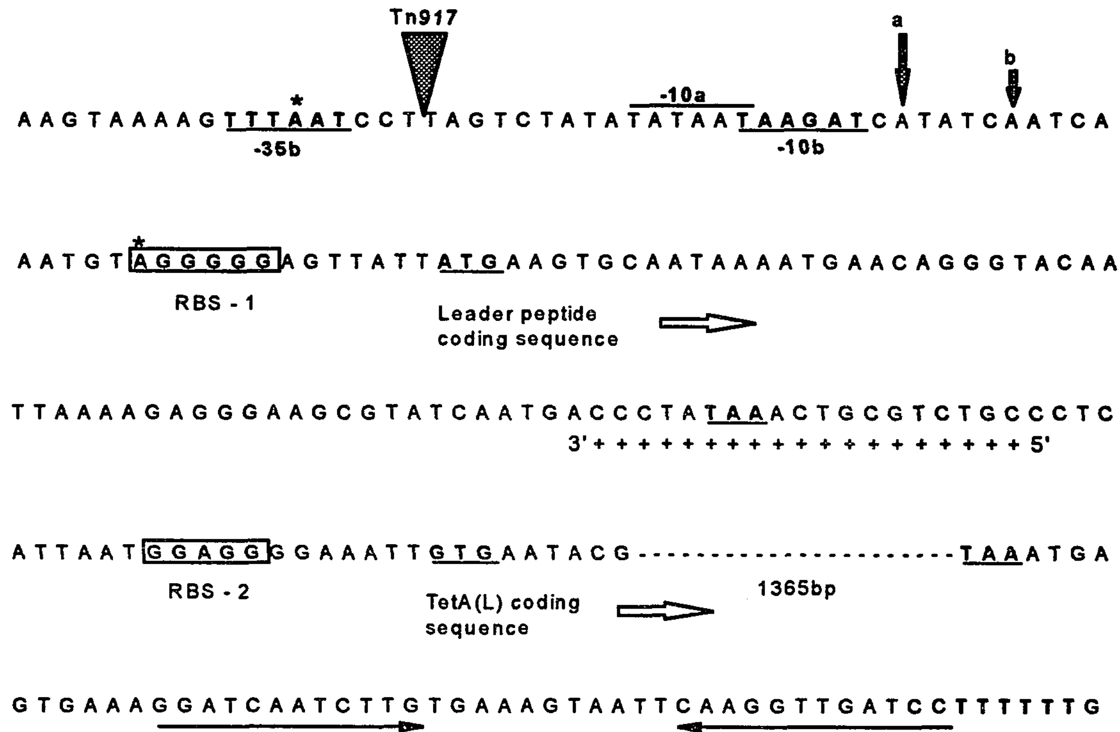


Figure 14. Nucleotide sequence of the *tetA*(L) transcription unit. Transcriptional start sites are marked by two arrows (a and b). Possible promoter sequences of -10 and -35 are indicated. Two ribosome binding sites for the leader peptide and *TetA*(L) are boxed, and the translational initiation and termination codons are underlined and in bold. The Putative rho-independent termination sequence is located downstream of the coding region, and its inverted repeat sequences are shown by two inverted arrows. The asterisks indicate the same positions of nucleotide bands shown in Figure 21. The sequences of the primer used in primer extension experiment are shown with plus signs.

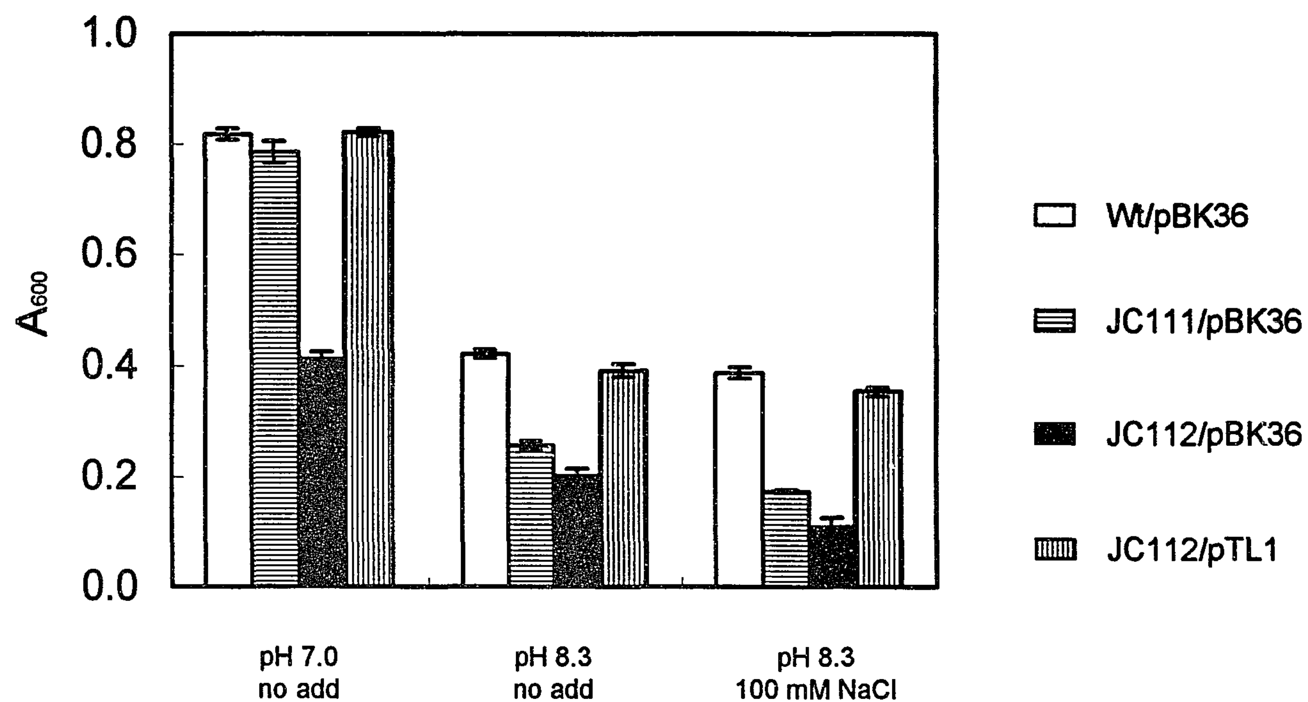


Figure 15. Growth of *B. subtilis* strains in TKM medium. All three strains were transformed with pBK36, and addtionally JC112 was transformed with pTL1 (cloned *tetA*(L)). All transformants were grown at pH 7.0 or pH 8.3. NaCl was added at the indicated concentration. The A_{600} was measured after 15 hours of growth.

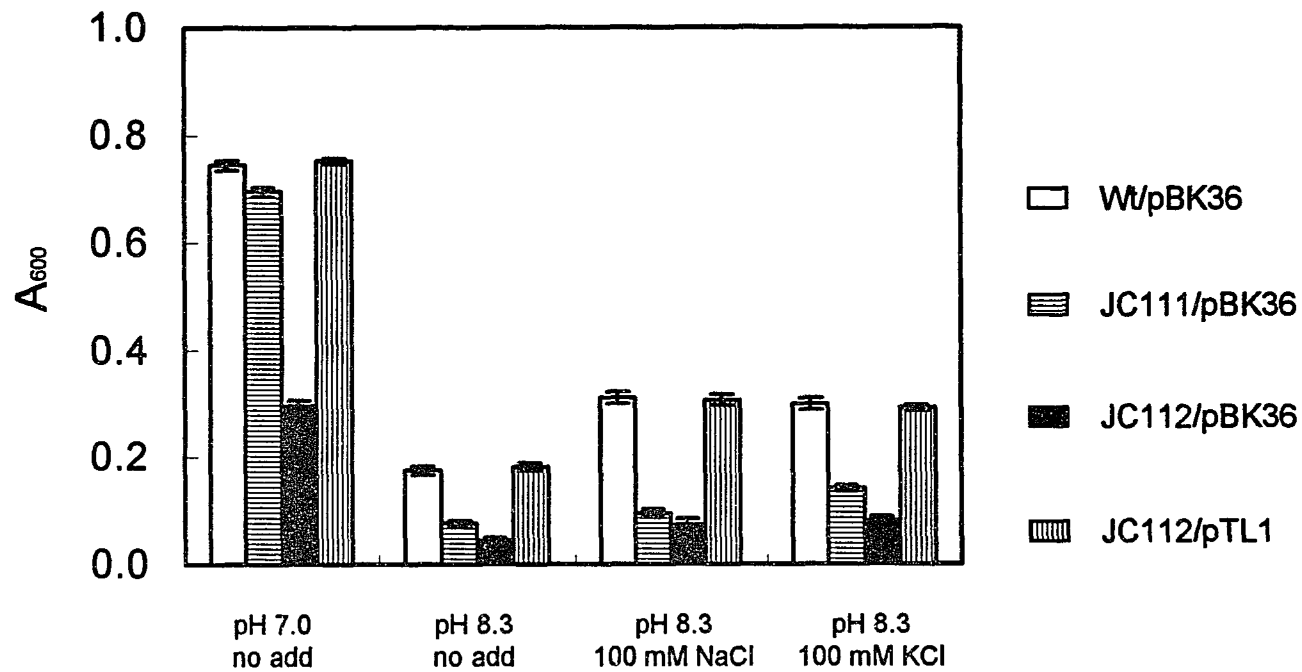


Figure 16. Growth of *B. subtilis* strains in TTM medium. All three strains were transformed with pBK36, and addtionally JC112 was transformed with pTL1 (cloned *tetA*(L)). All transformants were grown at pH 7.0 or pH 8.3. NaCl or KCl was added at the indicated concentration. The A_{600} was measured after 15 hours of growth.

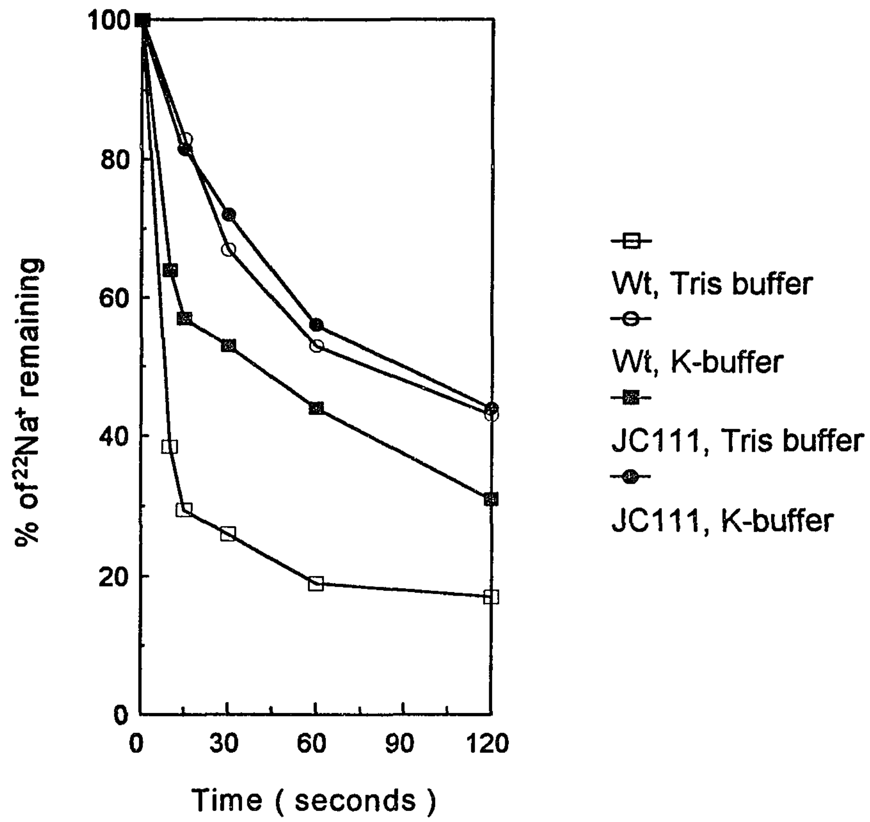


Figure 17. $^{22}Na^{+}$ efflux from wild type and JC111 cells energized by a valinomycin-mediated potassium diffusion potential. Cells were starved, loaded with $22Na^{+}$ and potassium, and treated with valinomycin. Efflux from wild type and JC111 was initiated by dilution into Tris-buffer or potassium buffer, and $^{22}Na^{+}$ inside of cells was measured at different time points.

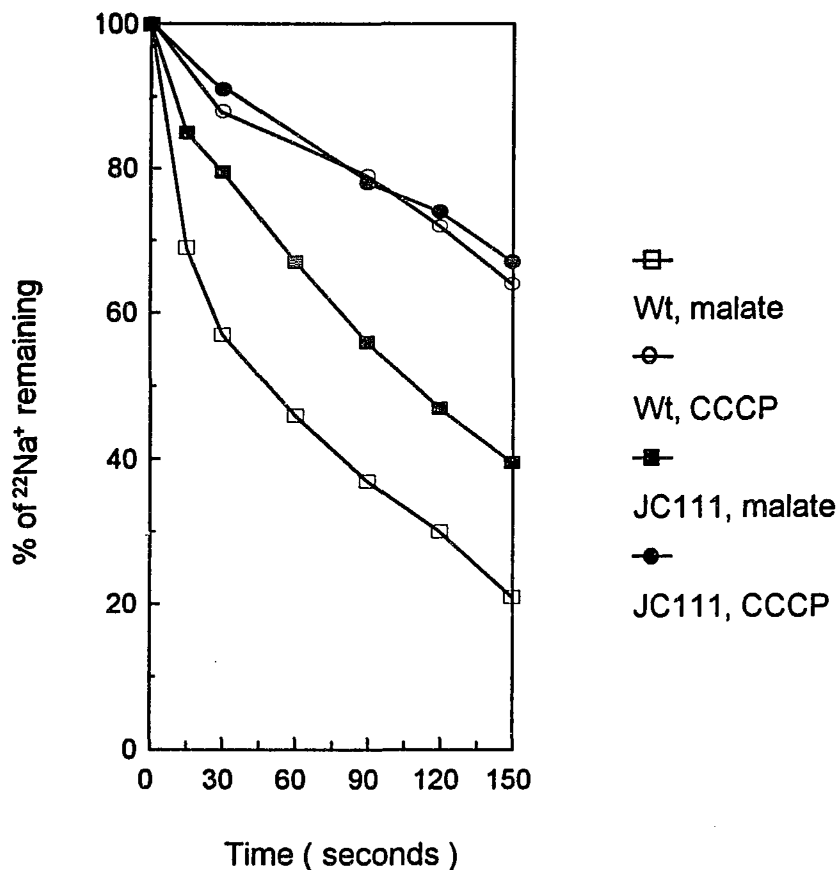


Figure 18. $^{22}Na^{+}$ efflux from wild type and JC111 cells energized by respiration. Cells were starved, loaded with $^{22}Na^{+}$. Where uncoupler was added, the samples were treated with 10 μM CCCP for 15 minutes prior to the dilution. Efflux from wild type and JC111 was initiated by dilution into 100 mM potassium phosphate buffer, pH 8.5, containing 10 mM malate and $^{22}Na^{+}$ inside of cells was measured at different time points.

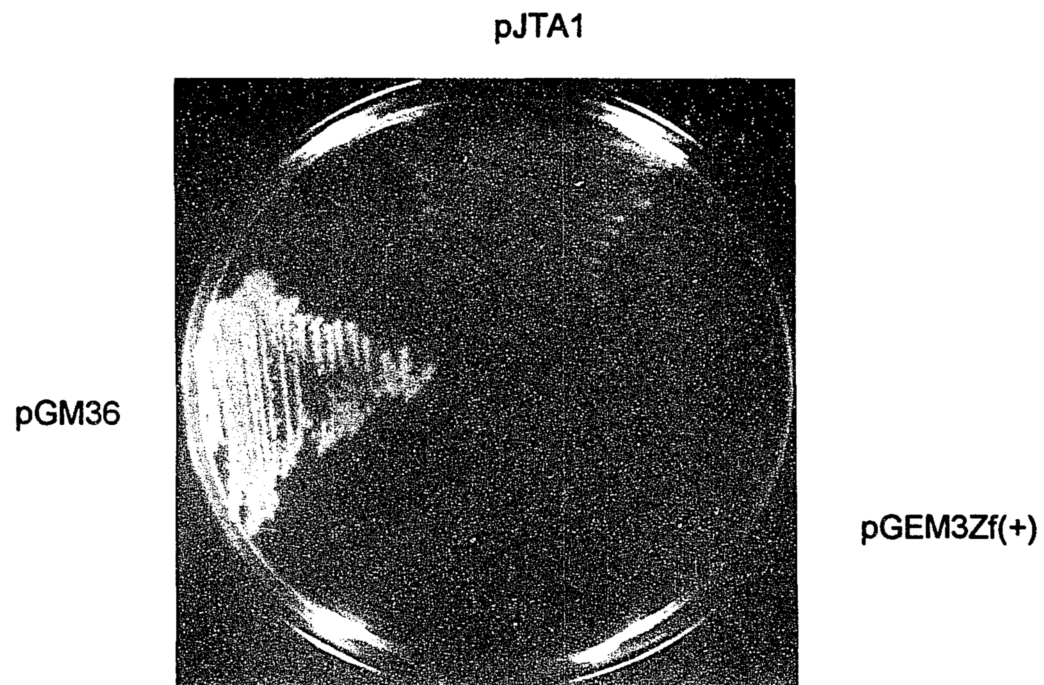


Figure 19. Growth of the transformants of *E. coli* strain NM81 on LBK/0.6 M NaCl plate. The NM81 strain was transformed with pGM36, pJTA1and pGEM3Zf(+). The transformants were isolated and re-streaked on an LBK/0.6 M NaCl plate. The cells were grown at 37°C overnight. Although not shown here, all three transformants grew equally well on LBK in the absence of NaCl.

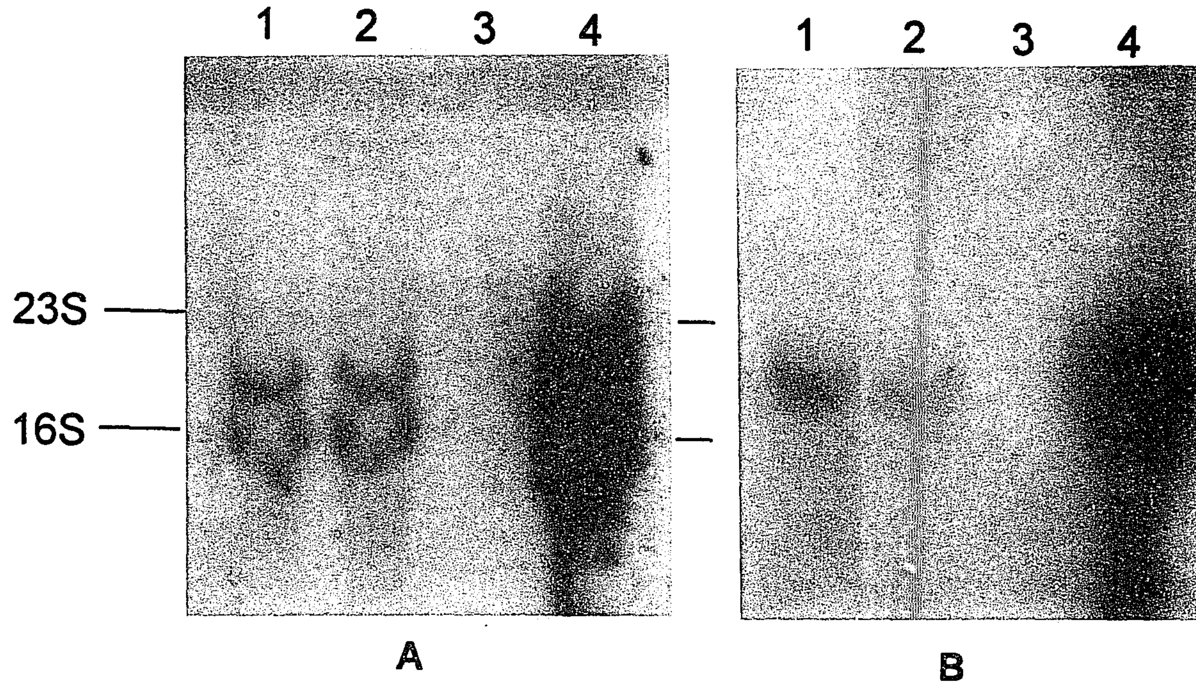


Figure 20. Northern blot analysis. 10 µg of total RNA prepared from wild type (lane 1), JC111 (lane 2), JC112 (lane 3) and JC111/pCIS7 (lane 4) was run on a 0.8% agarose gel. The samples were blotted onto a nylon membrane and probed with the R1 riboprobe (A) and R2 riboprobe (B).

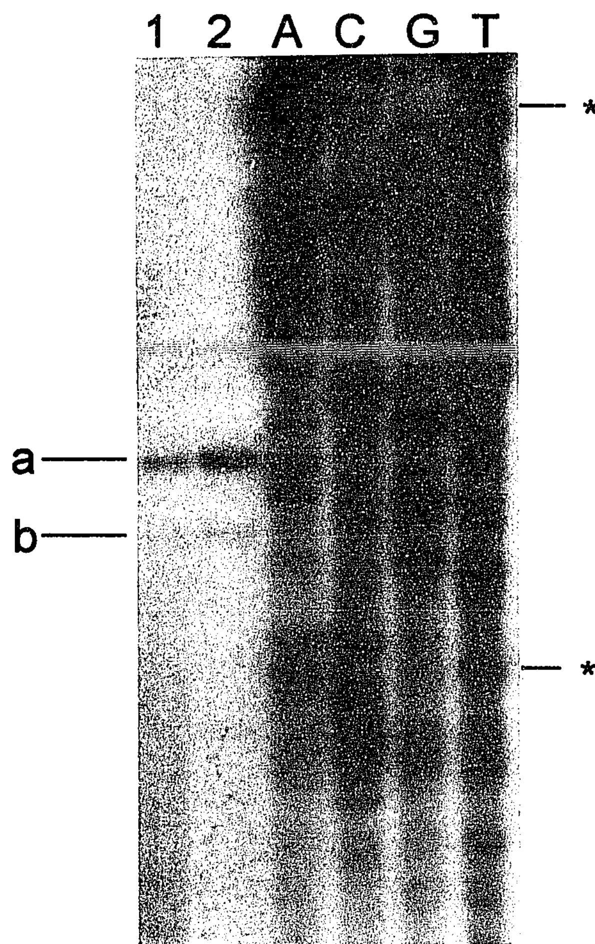


Figure 21. Primer extension mapping of transcriptional start sites. RNA was prepared from the wild type (lane 1) and JC111/pCIS7 (lane 2) strains. The entire reverse transcriptase reaction of wild type was loaded and only one-tenth of that of JC111/pCIS7 was loaded. The four right lanes are a dideoxy sequencing ladder of the*tetA*(L) leader region with the same oligonucleotide primer (shown in Figure 14) as was used in the reverse transcriptase reactions. Arrow a is the newly mapped mRNA start site with a strong signal. Arrow b indicates the start site which was mapped before in a plasmid derived *tet*(L). Bands labelled with asterisks indicated the positions of the nucleotides marked with asterisks in Figure 14.

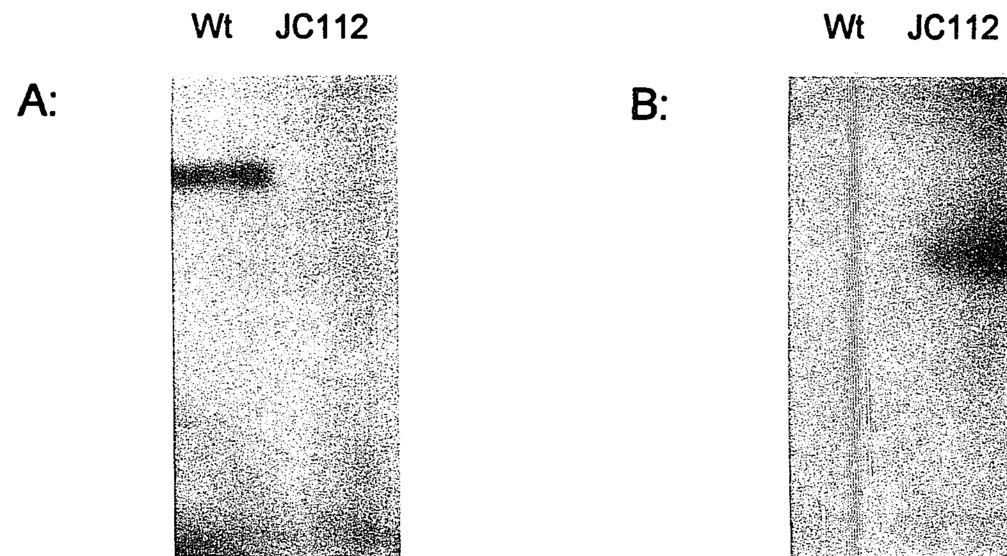


Figure 22. Southern analysis of the chromosomal DNA of wild type and JC112. 10 µg of DNA was digested with *EcoR*I. The samples were run on a 0.8% agarose gel and blotted. The probes used are PCR products derived from the *tetA*(L) gene coding region (A) and the cat coding region (B).

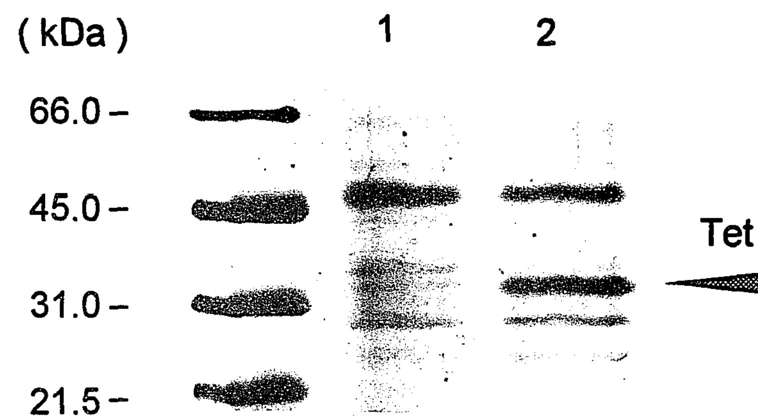


Figure 23. Overexpression of the TetA(L) protein. 10 µg of membrane proteins after initial purification by Ni-resin and EDTA from an uninduced sample (lane 1) and induced sample (lane 2) were electrophoresed on a single 10% SDS polyacrylamide gel. The protein bands were visualized by Coomassie staining. The arrow indicates the Tet protein band.

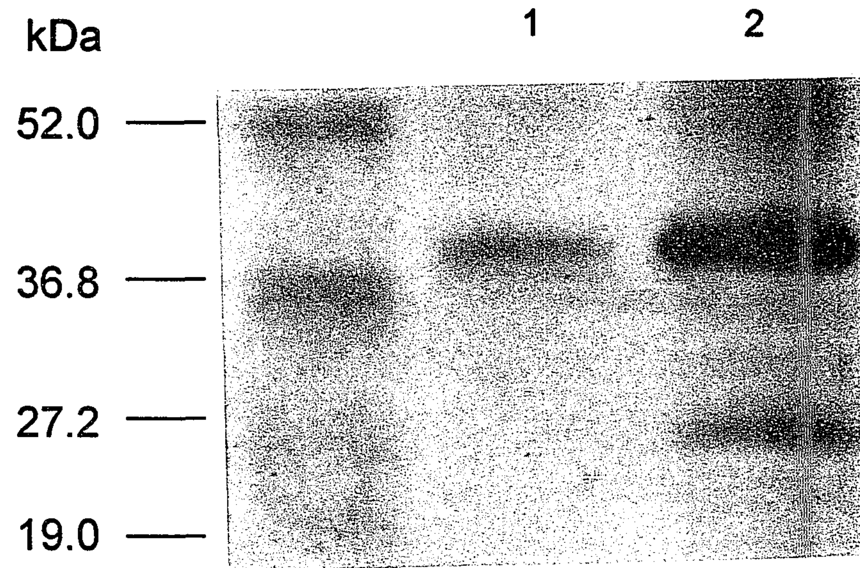


Figure 24. Western analysis of the Tet protein. 10 μg of membrane proteins after initial purification from the uninduced sample (lane 1) and the induced sample (lane 2) were electrophoresed on a 10% to 20% SDS polyacrylamide gradient gel and transferred onto a nylon membrane. An antibody prepared from rabbit against the N-terminal of the Tet protein was used to detect the sample. The color was developed by the alkaline phosphatase protocol (Bio-rad).

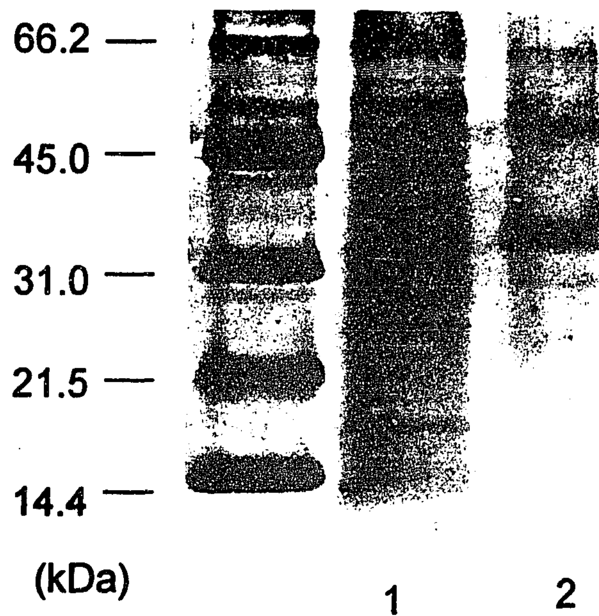


Figure 25. Purification of the TetA(L) protein. Proein samles from unbound portion of membrame extraction (lane 1, 5μg) and purified Tet fraction (lane2, 3μg) were electrophoresed on a single 10% SDS-polyacrylamide gel. The protein bands were visualized by coomassie staining.

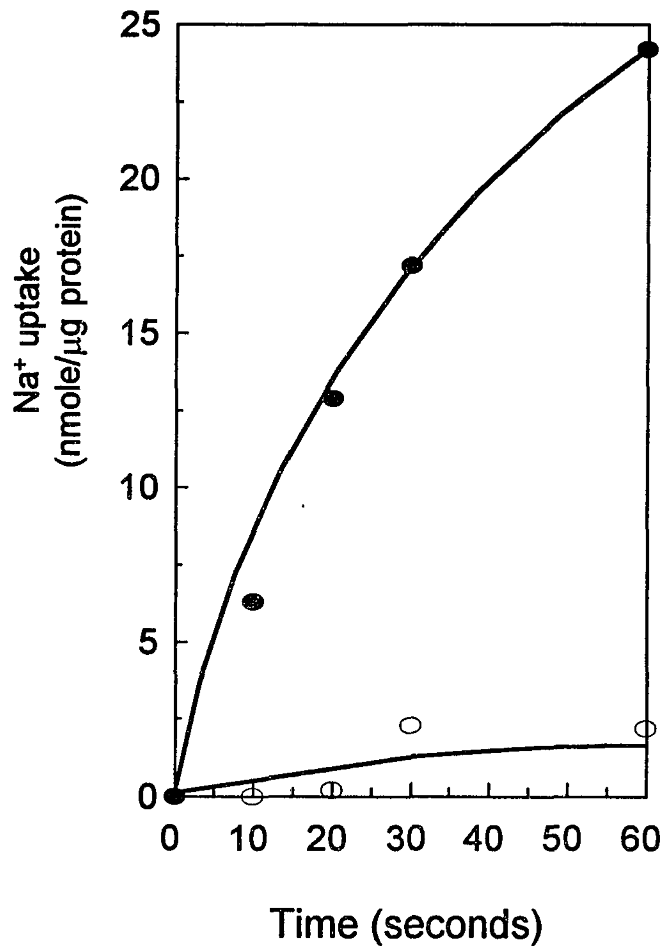


Figure 26. ΔpH-driven Na^+ uptake into proteoliposomes. The proteoliposomes reconstituted with purified TetA(L) were loaded with NH_4Cl. A pH gradient (acid inside) was imposed using ammonium chloride gradient and uptake of $^{22}NaCl$ was measured as described in Materials and Methods. The assay was started by dilution of 4 μl of proteoliposomes into a cholineCl buffer (●) or an NH_4Cl buffer (O) (control without ΔpH).

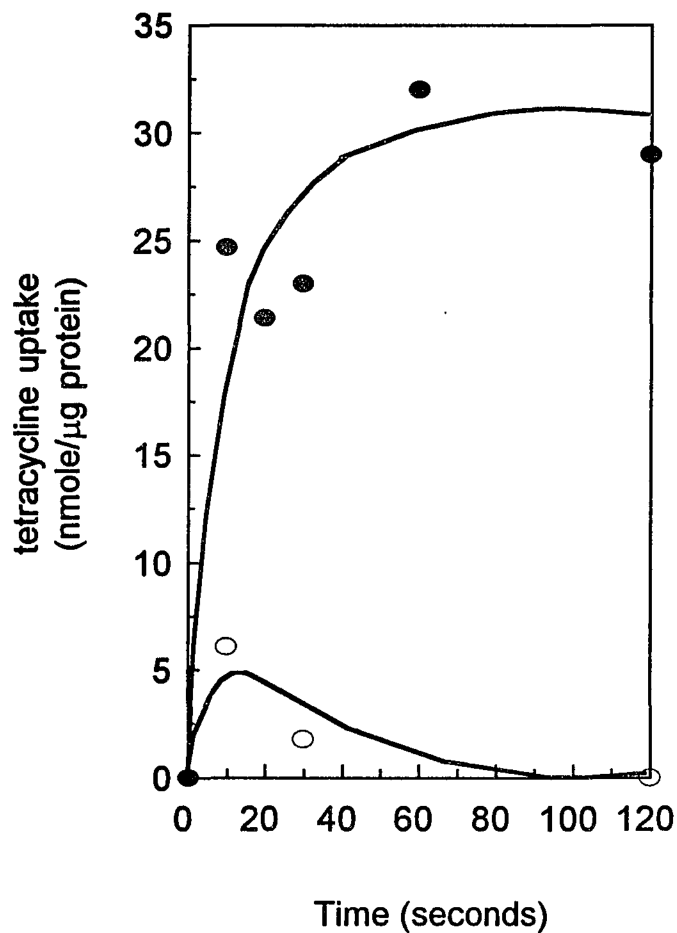


Figure 27. ΔpH-driven tetracycline uptake into proteoliposomes. The proteoliposomes reconstituted with purified TetA(L) were loaded with NH_4Cl. A pH gradient (acid inside) was imposed using NH_4Cl gradient. The uptake of [^{3}H]tetracycline was measured as described in Materials and Methods. The assay was started by dilution of 4 µl of proteoliposomes into a cholineCl buffer (●) or an NH_4Cl buffer (O) (control without ΔpH).

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