JOURNAL OF BACTERIOLOGY, Apr. 2000, p. 2088–2095 Vol. 182, No. 8
0021-9193/00/$04.00+0

Two Types of *Bacillus subtilis tetA*(L) Deletion Strains Reveal the Physiological Importance of TetA(L) in K^+ Acquisition as well as in Na^+, Alkali, and Tetracycline Resistance

WEI WANG,[1] ARTHUR A. GUFFANTI,[1] YI WEI,[1] MASAHIRO ITO,[2] AND TERRY A. KRULWICH[1]*

Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, New York, New York 10029,[1] *and Department of Life Science, Toyo University, Oura-gun, Gunma 374-0193, Japan*[2]

Received 3 December 1999/Accepted 20 January 2000

The chromosomally encoded TetA(L) protein of *Bacillus subtilis* is a multifunctional tetracycline-metal/H^+ antiporter that also exhibits monovalent cation/H^+ antiport activity and a net K^+ uptake mode. In this study, *B. subtilis* mutant strains JC112 and JC112C were found to be representative of two phenotypic types of *tetA*(L) deletion strains that are generated in the same selection. Both strains exhibited increased sensitivity to low tetracycline concentrations as expected. The mutants also had significantly reduced ability to grow in media containing low concentrations of K^+, indicating that the net K^+ uptake mode is of physiological consequence; the deficit in JC112 was greater than in JC112C. JC112 also exhibited (i) greater impairment of Na^+- or K^+-dependent growth at pH 8.3 than JC112C and (ii) a greater degree of Co^{+2} as well as Na^+ sensitivity. Studies were initiated to explore the possibility of two different patterns of compensatory changes in other ion-translocating transporters in these mutants. Increased expression of two loci has thus far been shown. Increased expression of *czcD-trkA*, a locus with a proposed involvement in K^+ uptake, occurred in both mutants. The increase was highest in the presence of Co^{2+} and was higher in JC112 than in JC112C. Deletion of *czcD-trkA* resulted in diminished growth of the wild-type and both mutant strains at low [K^+], supporting a significant role for this locus in K^+ uptake. Expression of *yheL*, which is a homologue of the Na^+/H^+ antiporter-encoding *nhaC* gene from *Bacillus firmus* OF4, was also increased in both *tetA*(L) deletion strains, again with higher up-regulation in JC112. The phenotypes resulting from deletion of *yheL* were consistent with a modest role for YheL in Na^+-dependent pH homeostasis in the wild type. No major role for YheL was indicated in the mutants in spite of the overexpression. The studies underscore the multiple physiological functions of TetA(L), including tetracycline, Na^+, and alkali resistance and K^+ acquisition. The studies also reveal and begin to detail the complexity of the response to mutational loss of these functions.

The chromosomal *tetA*(L) locus of *Bacillus subtilis* encodes a protein that confers resistance to low concentrations of tetracycline (Tc) by catalyzing efflux of a Tc-divalent metal ion complex in exchange for protons (Tc-Me^{+2}/H^+ antiport) (2, 8). Tc efflux does not occur in the absence of a divalent cation such as Co^{2+}, Mg^{2+}, or Mn^{2+}, with Co^{2+} being the best. Conversely, the divalent cation does not efflux via TetA(L) without Tc (9, 12, 32). Modest amplification of the gene or changes in the promoter region lead to increased expression and Tc resistance as does expression of the gene from a multicopy plasmid (4, 16). During the past few years, studies in this laboratory have established that TetA(L) is a multifunctional antiporter. In addition to electrogenic Tc-Me^{+2}/H^+ antiport, TetA(L) catalyzes the exchange of cytoplasmic Na^+ or K^+ for a greater number of external H^+ or K^+ ions (7–9, 12, 13). The monovalent cation/H^+ antiporter mode has roles in Na^+ resistance and pH homeostasis (8). While not yet demonstrated, the net K^+ uptake monovalent cation/K^+ antiporter mode could also be of physiological importance.

The enumeration and evaluation of the roles of TetA(L) have been complicated by the variable phenotype of isolates carrying the same *tetA*(L) deletion. Initial observations on these *tetA*(L) deletion strains, which focused on the most severe phenotype (e.g., mutant JC112), led to the suggestion that *tetA*(L) cannot be deleted without compensatory changes that might involve second-site mutation(s) (8). These studies also indicated that the TetA(L) efflux protein had an additional function that accounts for a growth deficit observed in *tetA*(L) mutants at pH 7 even in the absence of Tc or elevated Na^+ (8, 13). Initially, it was hypothesized that there might be an endogenous substrate for TetA(L) whose reduced efflux compromises growth (8). However, the more recent finding of the net K^+ uptake mode of TetA(L) (13) raises the possibility that inadequate K^+ acquisition is part or all of the basis for the growth defect of *tetA*(L) mutants at pH 7. The present study was directed toward categorization of the different phenotypes of *tetA*(L) deletion strains and clarification of the basis for the phenotype at pH 7. We further sought to begin the elucidation of the compensatory changes in *tetA*(L) deletion strains.

MATERIALS AND METHODS

Plasmids, bacterial strains, and growth conditions. The plasmids and *B. subtilis* strains used in this study are described in Table 1. The *tetA*(L) deletion mutants from the earlier study, including JC112 and JC112C, carried a chloramphenicol resistance (Cm^r) cassette which replaced the entire *tetA*(L) chromosomal coding sequence. The *tetA*(L) gene had been removed as a 1.8-kb *Cla*I-*Nde*I fragment in a previously described plasmid, which was then used to produce the mutants (8). One new *tetA*(L)-containing plasmid, pTL2, was used in this study. The coding sequence of *tetA*(L) was excised from pTL1 (8) by *Bam*HI-*Hin*dIII digestion and was cloned into the *Bam*HI and *Hin*dIII sites downstream of the *ermC* promoter of a pBK15 derivative named pVEB3 (Table 1). In this plasmid, an internal 750-bp *Mun*I fragment of the Cm^r gene of pBK15 was replaced with a 1.2-kb *Eco*RI fragment of pSP2 containing a spectinomycin resistance (Sp^r) gene. All new constructs were verified by sequence analysis. The sequencing was performed by the Utah State Biotechnology Center (Logan),

* Corresponding author. Mailing address: Box 1020, Department of Biochemistry and Molecular Biology, Mount Sinai School of Medicine, 1 Gustave L. Levy Place, New York, NY 10029. Phone: (212) 241-7280. Fax: (212) 996-7214. E-mail: terry.krulwich@mssm.edu.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
B. subtilis		
BD99	*hisA1 the-5 trpC2*	Wild type, from A. Garro
JC112	BD99 with *tetA*(L) deletion	8
JC112C	BD99 with *tetA*(L) deletion	8
BTK15	BD99 *amyE*::*yheL-lacZ*	This study
BTK16	JC112 *amyE*::*yheL-lacZ*	This study
BTK17	JC112C *amyE*::*yheL-lacZ*	This study
BTK18	BTK16 harboring *tetA*(L) gene containing plasmid pTL2	This study
BTK19	BTK17 harboring plasmid pTL2	This study
BTK21	BD99 *yheL*::Sp^r	This study
BTK22	JC112 *yheL*::Sp^r	This study
BTK23	JC112C *yheL*::Sp^r	This study
BTK30	JC112 harboring plasmid pAG4	D. Bechhofer
BTK32	JC112C harboring plasmid pAG4	This study
BTK33	BD99 *czcD*::Sp^r	This study
BTK34	JC112 *czcD*::Sp^r	This study
BTK35	JC112C *czcD*::Sp^r	This study
BTK38	JC112 *amyE*::*tetA*(L)	This study
BTK39	JC112C *amyE*::*tetA*(L)	This study
BTK40	BD99 containing plasmid pYheL-BK36	This study
BTK24	BD99 containing pBK36	This study
Plasmids		
pAC7	Integration vector at *amyE* locus	25
pAG4	*tetA*(L) gene cloned into pYH56	30
pBK15	pBD142-pBR322 joint replicon with multiple cloning sites downstream of *ermC* 5′ end	K. Zen
pBK36	pUB110-pBR322 joint replicon with multiple cloning sites downstream of *ermC* 5′ end	K. Zen
pVEB3	Replace a *Mun*I fragment internal of Cm^r in pBK15 with a Sp^r gene	This study
pGEM3zf+	Cloning vector	Promega
pGEM7zf+	Cloning vector	Promega
pAC7-K4L3	*yheL-lacZ* fusion plasmid for integration in *amyE* locus	This study
pCZCD2	5′ end of *czcD* gene cloned into pGEM3zf+	This study
pCZCD6	*czcD* operon disrupted with Sp^r gene cloned into pGEM3zf+; for knocking out *czcD*	This study
pNH2	*yheL* coding sequence disrupted with Sp^r gene cloned into pGEM7zf+; for knocking out *yheL*	This study
pYheL-BK36	*yheL* coding sequence cloned into pBK36	This study
pTL1	pBK36 harboring *tetA*(L) gene	8
pTL2	pVEB3 harboring *tetA*(L) gene from pTL1	This study
pTL6	*tetA*(L) cloned into pAC7 for integration in *amyE* locus	This study

using an ABI-100 model 377 sequencer. Construction of bacterial strains carrying deletions and/or gene fusions is described below.

Except where indicated, growth was conducted at 30°C. Liquid cultures were incubated with shaking. Media TKM and TTM are, respectively, K^+-replete and low-K^+ Tris-buffered media (both of which have no added Na^+ except where noted) (8). For experiments in which growth was measured in the presence of different concentrations of added K^+, the inoculum was grown for 15 h in modified TTM supplemented with sodium phosphate instead of potassium phosphate. Two-milliliter cultures of TTM-sodium phosphate with various amounts of added KCl were inoculated with 20 μl of the overnight cultures, and the A_{600} was measured after 8 h of incubation. SpizKM was used for studying $CoCl_2$ resistance because microprecipitation appeared to occur in the media buffered with Tris. SpizKM contains Spizizen salts (26), 50 mM potassium malate, 0.1% yeast extract, and 50 μg each of L-threonine, L-histidine, and L-tryptophan per ml. $CoCl_2$ was added as indicated. RNA preparation medium (6) was used for growth of cells for some of the Northern analyses.

Determination of MICs of Tc, Co^{2+}, and Na^+. To determine the MIC of Tc, cells were grown in TKM (pH 7.0) with various Tc concentrations. The A_{600} was measured after 17 h of growth. To avoid precipitation, the MIC for $CoCl_2$ was determined from the A_{600} of wild-type, JC112, and JC112C cells after 8 h of growth in SpizKM (pH 7.0) containing various $CoCl_2$ concentrations. For determination of the MIC for NaCl, TKM at pH 7.0 or 8.3 was supplemented with various concentrations of NaCl, and the A_{600} was measured after 15 h of growth. For each of these compounds or ions, the MIC was taken as the lowest concentration at which the A_{600} after the indicated period of growth was below 0.1.

Integration of a *tetA*(L) gene into the *B. subtilis amyE* locus. The *tetA*(L)-containing plasmid pAG4 (30) was digested with *Hin*dIII and then treated with mung bean nuclease. Subsequent digestion with *Cla*I released a 2.5-kb fragment containing *tetA*(L) and its promoter. This fragment was cloned into the *Sma*I and *Cla*I sites of pAC7 (25). The resulting plasmid, pTL6, was digested with *Nru*I and used to integrate the *tetA*(L) gene into the *amyE* loci of mutant strains JC112 and JC112C. The strains were identified by initial screening for kanamycin resistance (Km^r) followed by identification of starch-negative strains (25). Strains BTK38 and BTK39 were confirmed to be strains of JC112 and JC112C, respectively, which had incorporated the *tetA*(L) gene and promoter into the *amyE* locus.

Northern analyses. Total RNA isolation and Northern analyses were conducted as described previously (6) to assess whether mRNA levels for a variety of genes were elevated. Primers used in PCR to amplify fragments for the probes employed in the analyses are listed in Table 2. A 400-bp PCR fragment (primers yqkI1 and yqkI2) was used as a probe for *yqkI*; a 400-bp PCR fragment (primers yusP1 and yusP2) was used as a probe for *yusP*; an internal 270-bp *Hin*dIII fragment of the PCR product (primers ycnB1 and ycnB2) was used as a probe for *ycnB*; a 310-bp *Nru*I fragment of the PCR product (primers yhcA1 and yhcA2) was used as a probe for *yhcA*; a 700-bp *Sph*I fragment from the *mrpA* locus was used as a probe for *mrpA* (14); a 180-bp fragment of pTCC1-25 (5) was used as a probe for *yybF*. A 780-bp *Hin*cII fragment from the *yheL* coding sequence (Fig. 1A) was used to probe the expression of *yheL*, an *nhaC* homologue. A 680-bp PCR fragment (primers Czcd3 and Czcd7) was cloned into pGEM3Zf(+) (Promega) via the *Hin*dIII and *Bam*HI sites. The resulting plasmid, pCZCD2, was linearized by *Hin*dIII digestion and used as the DNA template for preparation of a riboprobe transcribed in vitro by T7 RNA polymerase. A 410-bp PCR fragment (primers yhaU1 and uhaU2) was used as a probe for *yhaU*; a 670-bp PCR fragment (primers ykqB1 and ykqB2) was used as a probe for *yqkB*; an internal 510-kb *Bcl*I-*Cla*I fragment of the PCR product (primers ykrM1 and ykrM2) was used as a probe for *ykrM*; a *Cla*I fragment (495 bp) from the 5′ end of a PCR product (primers yuaA2 and yubG2) was used as a probe for *yuaA* and *yubG*.

Deletion of *czcD-trkA* from the wild type, JC112 and JC112C. A fragment containing the putative *czcD-trkA* operon was amplified by PCR with primers Czd6 and Trka6 (Table 2). Primer Czcd6 contained additional nucleotides creating a *Hin*dIII site, and primer Trka6 contained additional nucleotides creating a *Kpn*I site. The PCR product was digested with *Hin*dIII and *Kpn*I and ligated into the *Hin*dIII and *Kpn*I sites of pGEM3Zf(+) (Promega), resulting in plasmid pCZCD5. A 0.3-kb *Mun*I fragment of pCZCD5, containing the 3′ end of *czcD* and the ribosome binding site and 5′ end of *yheL*, was replaced by a PCR-

TABLE 2. Oligonucleotides used in this study

Primer	Sequence[a]	Corresponding sequence (bp)	Gene and/or GenBank accession no.
yhaU1	5′-GGAGAGGGCGTGTGAGCCCGATGG	60419–60396	Z99109 BSUB0006
yhaU2	5′-CGAGATTGTGATGACGCCTGCG	60007–60028	
yqkI1	5′-GGACTTCTTTTGAAGGATGTAAG	58183–58161	*yqkI*, Z99116 BSUB0013
yqkI2	5′-CGACTGTTCCCCATGACGTTC	57781–57801	
yusP1	5′-CCCTTGACGGTACCATTGTCG	177094–177114	*yusP*, Z99120 BSUB0017
yusP2	5′-CCAGCCGCCGATTTGCGG	177491–177474	
ycnB1	5′-CCGGATCCTTGAACACAAGTATTGAACAA	34284–34264	*ycnB*, Z99106 BSUB0003
ycnB2	5′-TTGGATCCGCTAAATAGCGGGGCTC	32834–32853	
yhcA1	5′-AAGGATCCATGCATCTGTTAAAATTTCTG	174398–174418	*yhcA*, Z99108 BSUB0005
yhcA2	5′-AAGGATCCCTGTACAGATTAAGATTCC	176071–176053	
ykqB1	5′-CCGGATCCATGAAAAAAGAATTTGCGG	125146–125164	Z99111 BSUB0008
ykqB2	5′-CCCCAAGCTTATTTGGCTGCTATTTTG	125820–125804	
ykrM1	5′-CCGGATCCATGAGATTAAAGTTTGG	20742–20759	Z99111 BSUB0008
ykrM4	5′-CGCCGATGCTTTGAAAACAGG	21419–21399	
yuaA2	5′-GAAGATCTATGGGAAGAATTAAAAATAAG	189715–189734	Z99119 BSUB0016
yubG2	5′-CTAGATCTCATATCGAATCATAAAAAATCC	191776–191755	
K1	5′-GCGGGTCTGGGCGGGTCGATG	44687–44707	*yheK*, Z99109 BSUB0006
K2	5′-CCCATCTCCTCATCAGCCG	(minus strand) 45274–45293	
K4	5′-GGGGAATTCCGACCTAAAAAACCGTTC	(minus strand) 45071–45090	
L1	5′-GGATATGAGGTTCTACATG	44118–44146	*yheL*, Z99109 BSUB0006
L2	5′-GGGGATCCGCTTTTTACAGCGGGG	42841–42860	
YheL/BF1	5′-ATGCGGATCCATGGATTCTCAAAAAAAGCTGACG	44225–44203	
YheL/HR1	5′-AGCAAGCTTATTTTTTAACAAATCCAATC	42865–42886	
L3	5′-GGGGATCCTCATTGTCAGTTTCGAAAG	43329–43349	
L7	5′-TCCATTCAAGCGGAACGCC	43773–43791	
CzcD1	5′-CCGGATCCATGGGTCA*A*CAATCATAATGAAG (containing an extra *A*)	124646–124625	Z99117 BSUB0014
CzcD3	5′-CCCAAGCTTGATTGAAGAAAAGG	124913–124896	
CzcD6	5′-CCCCAAGCTTATAGAAGCTATAGGTGG	124570–124552	
CzcD7	5′-CCCGGATCCGCCACTCATCATAATCC	124230–124249	
TrkA6	5′-CCGGTACCTATAAAGTACAATCCTTCTAC	122676–122699	
TrkA7	5′-GCCCAAGCTTATTCACCGTTCATTTGT	122586–122604	

[a] Extra nucleotides (underlined) were added for inducing restriction sites.

amplified Sp^r gene, producing plasmid pCZCD6. This plasmid was linearized by *Hin*dIII digestion and used to transform the wild type, JC112, and JC112C into Sp^r strains BTK33, BTK34, and BTK35, respectively. The disruption of the *czcD* and *trkA* genes was confirmed by PCR, restriction analyses, and sequencing.

Analysis of the transcriptional start and promoter region of *B. subtilis yheL*. The SUPERSCRIPT preamplification system (GIBCO BRL, Life Technologies) and its published standard procedure were applied for mapping the transcription start point. For each reverse transcription reaction, 5 μg of total RNA isolated from the wild type or JC112 and 50 pmol of 5′-end labeled primer K1 or L1 (Fig. 1A and Table 2) were used. Half of each primer extension reaction was resolved on a 5% polyacrylamide-urea denaturing gel, along with a ^{32}P-labeled size marker. A band of about 200 nucleotides (nt) resulted from extension using primer K1, and a band of about 800 nt resulted from primer L1 (data not shown). The other half of the primer extension reaction from primer K1 was resolved on an 8 M urea–6% polyacrylamide gel along side a sequence standard prepared using the same primer, K1, and a template (fragment K2L7) prepared by PCR amplification using primers K2 and L7 (Table 2). Bands of the same size were detected in both the wild type and JC112 (Fig. 1C). Sequencing of promoter region of *yheL* was conducted in the wild type, JC112, and JC112C. Chromosomal DNA from each strain was used as a template for PCR amplification (primers K2 and L1 [Table 2]) of a segment containing the putative promoter region (Fig. 1A). Each PCR product was gel purified and then sequenced using primers L1 and K1 (Table 2). The sequence of the PCR products was identical in the three strains and corresponded to that in the *B. subtilis* genome database (GenBank accession no. Z99109 BSUB0006).

Construction of *yheL-lacZ* fusions in the wild type, JC112, and JC112C. For production of strains of the wild type, JC112, and JC112C with a *yheL-lacZ* fusion integrated into the *amyE* locus, a fragment containing the putative promoter region of the *yheL* operon, from 160 bp upstream of the mapped transcription start site to the codon for amino acid 299 of the *yheL*, was amplified by PCR with primers K4 and L3 (Fig. 1A; Table 2). Since there were no sequence differences in the *yheL* promoter regions among the three strains, only the chromosomal DNA isolated from the wild type was used as a template for the amplification. Primer K4 contains additional nucleotides for an *Eco*RI site, and primer L3 contains additional nucleotides for a *Bam*HI site. It was thus designed for insertion into plasmid pAC7 (25), which was used to integrate the fusion gene into the *amyE* locus. The PCR product, K4L3, was digested with *Eco*RI and *Bam*HI and then ligated into pAC7 at *Eco*RI and *Bam*HI sites, resulting in an in-frame fusion of K4L3 and the *lacZ* gene (starting with the first codon of the *lacZ* coding sequence) in pAC7. *Escherichia coli* DH5α containing this *yheL-lacZ* fusion plasmid, pAC7-K4L3, formed blue colonies on Luria-Bertani plates with additions of 0.2 mM isopropylthio-β-D-galactoside (IPTG) and 0.04% (wt/vol) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal). Plasmid pAC7-K4L3 was digested with restriction enzyme *Nru*I. The linearized pAC7-K4L3 was transformed into the wild type, JC112, and JC112C, and Km^r, starch-negative strains were isolated and verified from each starting strain; BTK15, BTK16, and BTK17 were, respectively, derivatives of the wild type, JC112, and JC112C.

Assays of β-galactosidase activities of strains expressing *yheL-lacZ* fusions. Expression of *yheL-lacZ* fusion protein in strains BTK15, BTK16, and BTK17 and derivatives thereof was studied by analyzing β-galactosidase activity, as described previously (8). β-Galactosidase activity was expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per A_{600} (Table 4). Overnight cultures grown in the media specified in Table 4 were used in the β-galactosidase activity assay.

Deletion of the *yheL* gene. First a fragment containing the *yheL* operon was amplified by PCR with primers K4 and L2 (Table 2; Fig. 1A). Primer K4 contains additional nucleotides for an *Eco*RI site, and primer L2 contains additional nucleotides for a *Bam*HI site. The PCR product K4L2 was digested with *Eco*RI and *Bam*HI and ligated into pGEM7Zf(+) (Promega) in the *Eco*RI and *Bam*HI sites, resulting in plasmid pNH1. The 1-kb *Hin*dIII-*Pst*I fragment of pNH1 containing the ribosome binding site and 5′ end of *yheL* was replaced by a PCR-amplified Sp^r gene, producing plasmid pNH2. Plasmid pNH2 was digested with *Xho*I, and the linearized plasmid was then used to transform the wild type, JC112, and JC112C into Sp^r strains BTK21, BTK22, and BTK23, respectively. The disruption of *yheL* in the these strains was confirmed by PCR and restriction digestion analyses.

RESULTS

Phenotypes of the *tetA*(L) deletion strains. At the start of the study, six *tetA*(L) deletion strains that had been isolated earlier (8) were compared to the wild-type strain with respect to both MICs for Na^+ and Tc and pH profiles for growth. All of the strains are identical in phenotype either to JC112 or to JC112C, which are distinct, stable phenotypic types; both types are often represented in a particular deletion experiment, but the JC112C type predominates and is sometimes the only phe-

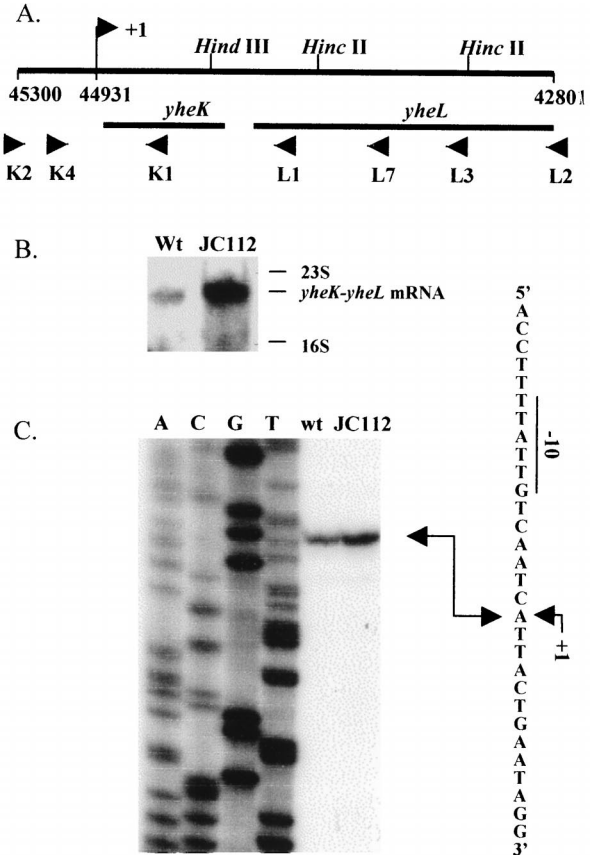

FIG. 1. (A) Schematic diagram of the *B. subtilis yheKL* region. This 2.5-kb fragment represents a reverse strand of nt 42801 to 45300 as reported in the *B. subtilis* genome project (GenBank accession no. Z99109 BSUB0006). The locations and relative sizes of the *yheK* and *yheL* coding sequences, and the oligonucleotides used in this study, are as indicated. The transcription start point mapped in this study is represented by an arrow at nt 44931. (B) Northern analysis of *yheL* in *B. subtilis* wild type (Wt) and JC112. A 780-bp *Hinc*II fragment from the *yheL* coding sequence (Fig. 1A) was ^{32}P labeled and used as the probe. The positions of 23S and 16S rRNAs are indicated on the right. (C) Reverse transcription mapping of the *yheL* transcription start site. The RNA used in the primer extension reactions was isolated from wild type or JC112. Control lanes (labeled A, C, G, and T) are a DNA sequence ladder using the same primer K1 as used in primer extension. The complement of the sequence that contains the mapped transcription start site (+1) and −10 are presented on the right.

notype formed. JC112 and JC112C were chosen for the studies reported here. First, the MICs for Tc, Na^+, and Co^{2+} were determined. A Tc- and Na^+-sensitive phenotype was expected, given the known functions of TetA(L). Both JC112 and JC112C were more sensitive than the wild type to low concentrations of Tc, exhibiting MICs of 0.09 and 0.12 μg/ml, respectively, versus 1.4 μg/ml for the wild type (Table 3). The expected Na^+ sensitivity, by contrast, was exhibited by only one of the *tetA*(L) mutants, JC112, which showed elevated relative sensitivity even at neutral pH, where Na^+ is less cytotoxic than at pH 8.3 (Table 3). JC112C did exhibit a modestly compromised ability to use either Na^+ or K^+ to support growth at pH 8.3, relative to the wild type, but JC112 exhibited a much more pronounced phenotype under such conditions (data not shown). Earlier work showed that this characteristic correlates with $Na^+(K^+)/H^+$ antiport status in support of pH homeostasis at elevated pH (8).

Co^{2+} toxicity was of interest because this is the optimal cation for the Tc-divalent cation complex that effluxes via

TABLE 3. MICs of Tc and $CoCl_2$ for wild-type *B. subtilis*, JC112, and JC112C and derivatives thereof

Strain	MIC (mean ± SD)			
	$CoCl_2$ (μM)[a]	Tc (μg/ml)[b]	Na^+ (M)[c]	
			pH 7.0	pH 8.3
BD99	425 ± 15	1.4 ± 0.13	1.5 ± 0.12	0.7 ± 0.05
JC112	150 ± 10	0.09 ± 0.01	0.9 ± 0.10	0.4 ± 0.06
JC112C	310 ± 25	0.115 ± 0.01	1.4 ± 0.09	0.7 ± 0.06
BTK38	125 ± 10	1.4 ± 0.1	1.3 ± 0.11	0.7 ± 0.04
BTK39	350 ± 20	1.4 ± 0.1	ND[d]	ND
BTK33	230 ± 15	1.8 ± 0.2	1.4 ± 0.10	0.8 ± 0.06
BTK34	140 ± 15	0.09 ± 0.01	0.9 ± 0.07	0.4 ± 0.08
BTK35	225 ± 20	0.105 ± 0.05	1.4 ± 0.08	0.8 ± 0.05

[a] Minimal concentration at which growth in SpizKM (A_{600}) was below 0.1 after 8 h at 30°C.
[b] Minimal concentration at which growth in TKM (A_{600}) was below 0.1 after 17 h at 30°C.
[c] Minimal concentration at which growth in TKM (A_{600}) was below 0.1 after 17 h at 30°C.
[d] ND, not determined.

TetA(L) (12, 31). Thus, if an endogenous substrate, e.g., an antibiotic produced by *B. subtilis*, normally exits by TetA(L)-mediated efflux in complex with Co^{2+} just as Tc does, then a *tetA*(L) deletion strain would be more sensitive than the wild type to growth inhibition by Co^+ even when Tc is absent. As shown in Table 3, both JC112 and JC112C were more sensitive than the wild type to inhibition by Co^{2+}. JC112 was significantly more sensitive to Co^{2+} than JC112C, even though their Tc sensitivities were comparable. While the Co^{2+} data shown were from experiments conducted in SpizKM, the same relative pattern among the strains was observed in TKM (data not shown). Also, although not shown, when the MICs were determined at 37°C rather than 30°C, all were unchanged except in the case of JC112C, which was consistently found to have an MIC for Co^{2+} that was higher, at 600 μM, than that of any of the other strains at either temperature. JC112C appears to have a mechanism that counteracts the increased Co^{2+} sensitivity that accompanies mutational loss of *tetA*(L). This mechanism appears to minimize the sensitivity relative to JC112 at 30°C and more than compensate at 37°C, e.g., by altered expression of a temperature-dependent regulator of a Co^{2+} efflux system.

It was of interest to explore whether the Co^{2+} sensitivity that is exhibited by both JC112 and JC112C at 30°C was a direct consequence of the loss of TetA(L) function. This would be

TABLE 4. β-Galactosidase levels in *B. subtilis* BTK15, BTK16, and BTK17

Growth pH	Medium	0.1 M NaCl	β-Galactosidase activity (nmol/min/A_{600}; mean ± SD)		
			BTK15	BTK16	BTK17
7.0	TTM	−	17.0 ± 1.6	125 ± 12.8	96.5 ± 8.2
		+	23.7 ± 3.5	130 ± 14.8	111 ± 4.2
	TKM	−	21.9 ± 1.0	186 ± 18.3	111 ± 9.1
		+	28.2 ± 3.4	145 ± 7.4	94.7 ± 12.5
8.0	TTM	−	18.6 ± 3.6	135 ± 9.5	75.3 ± 6.5
		+	22.7 ± 3.5	138 ± 12.5	91.3 ± 6.9
	TKM	−	23.2 ± 2.1	213 ± 24.0	94.7 ± 4.5
		+	49.6 ± 11.4	307 ± 22.0	119 ± 15.2
7.0	SpizKM	−	70.6 ± 7.5	228 ± 30.9	148 ± 11.3
	SpizKM + 50 μM $CoCl_2$	−	168 ± 20.5	224 ± 42.6	177 ± 6.6

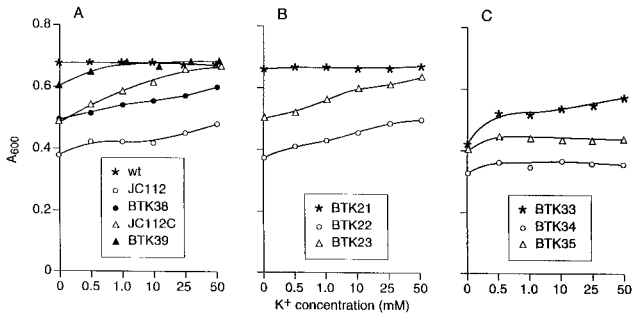

FIG. 2. Growth of wild-type (wt) and mutant strains of *B. subtilis* at various concentrations of added K^+. The cells were grown in modified TTM (pH 7.0) and adjusted to the indicated concentrations of K^+ added to the medium (see Materials and Methods). The A_{600} was measured after 15 h of growth at 30°C.

consistent with an involvement of TetA(L) in efflux of a Co^{2+} endogenous substrate complex. Alternatively, Co^{2+} sensitivity could be a secondary consequence of the *tetA*(L) disruption. Reintroduction of an active *tetA*(L) gene should reverse the Co^{2+} sensitivity if it is a direct consequence of functional TetA(L) loss and might even reverse sensitivity that was secondary to TetA(L) loss. As anticipated, reintroduction of the *tetA*(L) gene in single copy, under its own promoters, in the *amyE* locus restored the Tc resistance of both JC112 (BTK38) and JC112C (BTK39) and the Na^+ resistance of JC112 (BTK38) to wild-type levels (Table 3). However, the enhanced Co^{2+} sensitivity of both JC112 and JC112C remained upon reintroduction of the functional *tetA*(L) (Table 3) and was even retained when a multicopy plasmid bearing *tetA*(L) was expressed in JC112 (data not shown). Moreover, JC112 and to a lesser extent JC112C, into which an active *tetA*(L) was reintroduced, retained some of the characteristic growth deficit at pH 7.0 in the absence of added Na^+ and Tc (Fig. 2; see below). Therefore, it is unlikely that the growth deficit at pH 7.0 is related to adverse accumulation of an endogenous substrate such as an antibiotic or that the increased Co^{2+} sensitivity of *tetA*(L) deletion mutants relates to a coupling of Co^{2+} efflux to such an endogenous substrate of TetA(L).

Whatever the basis for the increased Co^{2+} sensitivity might be, it raises the possibility that the growth defect of *tetA*(L) deletion mutants at pH 7 in the absence of added Tc or Na^+ is attributable to an inhibition of growth by accumulation of toxic divalent cations from normal growth media. Alternatively or in addition, the net K^+ uptake mode of TetA(L) may have physiological significance, and the growth defect at pH 7 arises from a diminished capacity for K^+ acquisition. As shown in Fig. 2A, both JC112 and JC112C exhibited less growth than the wild type in media containing only contaminating levels of K^+. With the addition of increasing [K^+] to the media, the growth of JC112C reached that of the wild type at 50 mM added K^+. In strain JC112C, into which a single active *tetA*(L) had been reintroduced (BTK39), there was still a slight deficit in growth at no added K^+, but wild-type growth levels were reached at 1 mM added K^+. With the more severe phenotype of JC112, growth was enhanced only modestly as [K^+] was increased, even to 50 mM. In addition, whereas reintroduction of an active *tetA*(L) (BTK38) markedly improved the growth profile at increasing [K^+], the growth level never reached that of the wild type. Quite possibly, in the more Co^{2+}-sensitive JC112, the growth deficit at pH 7 results from a combination of a major deficit in K^+ acquisition, especially at low [K^+], and a significant contribution of overaccumulation of toxic divalent cations from the medium. The small residual deficit in growth of JC112C with restored *tetA*(L) (BTK39), at no added K^+, could similarly reflect a contribution, albeit smaller, of toxic divalent cation overaccumulation to the pH 7.0 phenotype.

Preliminary Northern analyses. From BLAST analyses (1), selection was made of candidate genes that might compensate for different functions of TetA(L). These genes, for which preliminary Northern analyses were conducted, were chosen from three different groups: (i) *ycnB*, *yhcA*, *yusP*, and *yybF*, four genes whose predicted products have sequence similarity with *tetA*(L); (ii) *mrp* (14, 18), a gene locus with known Na^+/H^+ antiporter activity, and three genes that had sequence similarity to Na^+/H^+ antiporter-encoding genes from other bacteria, *yheL* and *yqkI*, which had similarity to the *nhaC* gene from alkaliphilic *Bacillus firmus* OF4 (14, 17), and *yhaU*, which had sequence similarity to the *napA* gene from *Enterococcus hirae* (31); and (iii) a gene locus for which there is a putative K^+ uptake function, *czcD-trkA* (28), and three genes or gene loci that show homology to one or both of the genes of the K^+ uptake-encoding *ktrAB* locus of *Vibrio alginolyticus* (20), *ykqB*, *ykrM*, and *yuaA-yubG*. Bands of the anticipated size were observed on Northern blots probed for each of these genes or loci (data not shown). The mRNA abundance was clearly elevated for two of them, and for those two loci the increase in mRNA was evident in both JC112 and JC112C. The two loci whose mRNA levels were elevated in JC112 and JC112C were the *czcD-trkA* locus that had been proposed to be involved in K^+ uptake (28) and the *nhaC* homologue *yheL*.

Role of *czcD-trkA* in the wild-type and *tetA*(L) deletion strains. The *B. subtilis czcD* gene, whose product is 42% identical to that of *Ralstonia* (previously *Alcaligenes*) *eutrophus* (21), is in an apparent operon with a gene that we designated as *trkA* (28). This locus was identified in our laboratory in an earlier screen of *B. subtilis* genes that increased K^+ acquisition by a K^+ uptake-deficient *E. coli* strain. Although the *B. subtilis trkA* gene product exhibits only partial sequence similarity to small regions of the TrkA component of an *E. coli* K^+ uptake system, the *B. subtilis trkA* alone complemented an *E. coli* carrying a mutation in its own *trkA* (28). The elevation of *czcD-trkA* expression in the *tetA*(L) deletion mutants thus appeared consistent with a compensatory change for purposes of K^+ acquisition. Northern analyses (Fig. 3) indicated that (i) the *czcD-trkA* mRNA abundance is low in the wild type grown on SpizKM at pH 7.0; (ii) this basal level of mRNA abundance is elevated in both JC112 and JC112C, and the increase is not abolished upon reintroduction of a functional *tetA*(L) gene into the two mutant strains (BTK38 and BTK39, respectively); (iii) all strains had a much higher level of *czcD-trkA* mRNA when grown in the presence of Co^{2+}, in the rank order BTK38 [JC112 with *tetA*(L) restored] > BTK39 [JC112C with *tetA*(L) restored] > JC112 > JC112C ≫ wild type.

To assess whether the *czcD-trkA* locus was related to K^+ acquisition or Co^{2+} sensitivity in vivo, mutants of the wild type, JC112, and JC112C (BTK33, BTK34, and BTK35, respec-

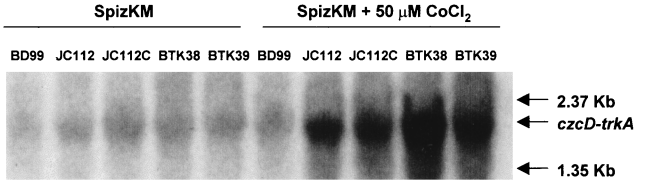

FIG. 3. Northern analysis of *czcD* mRNA in the wild type, JC112, JC112C, and the two mutant strains upon restoration of an active *tetA*(L) gene, BTK38 and BTK39, respectively. The cells were grown in SpizKM with or without added $CoCl_2$ as shown.

tively) were studied. As shown in Fig. 2C, in comparison with Fig. 2A, deletion of both the *czcD* and *trkA* genes from the wild type resulted in a growth deficit at no added K^+. Growth of BTK33 at no added K^+ was comparable to that exhibited by JC112 carrying no additional mutations. At higher K^+ concentrations, BTK33 showed growth somewhat better than that of JC112 but still much less than the wild-type level. The two mutants also showed a more severe phenotype after *czcD-trkA* disruption. They exhibited reduced growth in medium with no added K^+ and responded only slightly to addition of K^+. These data support a role for this locus in K^+ acquisition and for the hypothesis that *czcD-trkA* elevation upon *tetA*(L) deletion provides partial compensation for a reduction in this capacity.

As shown in Table 3, deletion of the *czcD* and *trkA* genes increased the Co^{2+} sensitivity of the wild type and JC112C. This is consistent with the finding that the *Ralstonia* CzcD mediates exclusion of Co^{2+} (3). JC112, whose Co^{2+} sensitivity was very high, did not exhibit a statistically significant increase in sensitivity. These findings are not consistent with overexpressed CzcD-TrkA causing increases in both K^+ and Co^{2+} uptake that underly the increased sensitivity of JC112 and JC112C to Co^{2+}. Some other basis for the enhanced Co^{2+} sensitivity must exist in the *tetA*(L) mutants. Neither the Tc nor Na^+ sensitivities of the three strains was altered by introduction of the *czcD-trkA* mutation (Table 3).

Expression and role of *yheL* in the wild-type and *tetA*(L) deletion strains. The putative antiporter-encoding *yheL* gene is downstream of, and would appear to form a likely operon with, *yheK*, a gene that exhibits sequence similarity to diverse regulatory genes (Fig. 1A). The size of the RNA detected with a *yheL* probe was consistent with this expectation, as shown in Fig. 1B, and a transcriptional start was mapped upstream of *yheK* (Fig. 1A and C). The elevated level of *yheL* RNA in JC112 relative to the wild type is evident in Fig. 1B. JC112C showed a more variable elevation of *yheL* mRNA in Northern experiments but had consistently more *yheL* RNA than the wild type; moreover, the elevated levels of *yheL* RNA of both mutant strains remained higher than in the wild type upon restoration of a functional *tetA*(L) either in single copy or on a multicopy plasmid (data not shown).

To better assess possible differences in *yheL* expression between JC112 and JC112C in the face of variable results of Northern analyses, we undertook experiments using *yheL-lacZ* fusions that monitored expression from the *yheL* promoter. The wild-type and mutant promoter regions were first shown, by sequencing, to be identical (see Materials and Methods). The expression of *yheL* was examined via β-galactosidase activity assay under various conditions. As shown in Table 4, (i) there was a striking increase in β-galactosidase activity in BTK16 (JC112) and BTK17 (JC112C) over BTK15 (wild type) under all conditions, and the activity in BTK16 was consistently and significantly greater than that in BTK17; (ii) in BTK16 (JC112) in particular, K^+-replete conditions (TKM or SpizKM versus TTM) favored higher *yheL* expression; (iii) small effects of added NaCl on *yheL* expression were observed only in the wild type at elevated pH and on TKM; and (iv) in K^+-replete SpizKM, in which Co^{2+} effects are best studied, *yheL* expression by BTK15 (wild type) was enhanced in the presence of $CoCl_2$. The major findings confirmed the indications from Northern analyses of greatly elevated *yheL* expression in JC112C and, even more, in JC112.

To confirm that *yheL* product functions as a Na^+/H^+ antiporter of the *nhaC* type, and to assess whether its elevated expression in JC112 and JC112C was essential for viability of these *tetA*(L) deletion strains, *yheL* was deleted from the wild type, JC112, and JC112C as described in Materials and Methods. The successful construction of these strains, BTK21, BTK22, and BTK23, respectively, showed that the two *tetA*(L) deletion strains were not dependent on *yheL*. The growth response of these double-deletion strains to various concentrations of K^+ was not different from the response of strains prior to *yheL* deletion (Fig. 2B), and the MICs for Tc, Na^+, and Co^{2+} were also indistinguishable from those of the initial strains (data not shown). The NhaC of *B. firmus* OF4 is an antiporter with a high Na^+ affinity that has little impact on Na^+ resistance per se. It does have a modest role in Na^+-dependent pH homeostasis (15). Prior studies have indicated that Na^+-dependent growth stimulation in low-K^+ medium (TTM) at pH 8.3 reflects this function (8, 19). Consistently, the Na^+-dependent stimulation of growth yield of the wild type, when challenged by elevated pH, was reproducibly diminished in its *yheL* derivative (BTK21) (data not shown).

The complex response of *B. subtilis* to *tetA*(L) deletion apparently involves increased expression of multiple membrane-associated proteins that is not reversed upon reintroduction of *tetA*(L). This suggested the possibility that the increased Co^{2+} sensitivity of such mutants might be a secondary by-product of this complex response in which the large increase in aggregate or some specific membrane proteins leads to a compromise in the membrane barrier to Co^{2+}. As an initial assessment of such a hypothesis, wild-type cells were transformed with the vector pBK36 (BTK24) or with pBK36 expressing *yheL* (BTK40). Whereas the control vector did not change the MIC for Co^{2+} of the wild-type strain, the expression of *yheL* resulted in a markedly increased sensitivity such that BTK40 was sensitive to as little as 100 μM $CoCl_2$ (data not shown).

DISCUSSION

A major finding of this study is that the net K^+ uptake mode (13) of the chromosomally encoded TetA(L) protein plays a significant physiological role in K^+ acquisition by *B. subtilis*. The growth deficit of *tetA*(L) deletion strains, in the absence of a challenge by alkali, Na^+ and Tc, correlates qualitatively with their [K^+]-related growth profile. A small growth deficit in JC112C, and larger component in JC112, is not abolished when a functional *tetA*(L) is restored to these mutants. This irreversible component of the mutants' phenotype is probably an indirect result of a pattern of changes in the expression of other genes. Complex patterns of response to *tetA*(L) deletion are the other major finding of these studies. The complexity may relate to the diverse functions of TetA(L).

This study showed that *tetA*(L) deletion strains exhibit two, partially distinct patterns of change in the expression of other genes. Two genes whose expression is significantly elevated have been identified in both phenotypic types of *tetA*(L) deletion mutants, but the catalogue is almost certainly incomplete. We hypothesize that there may be a regulon whose member genes encode ion-translocating transporters with roles in monovalent cation, pH, and perhaps divalent cation homeostasis. The two gene loci found here to be up-regulated in JC112 and JC112C, i.e., *czcD-trkA* and *yheL*, would be members of this putative regulon. Another member is probably *tetA*(L) itself. It was earlier noted that the basal level of expression of a *tetA*(L)-*lacZ* fusion was markedly increased in JC112 (8). A unifying hypothesis would be that the putative regulon has one or more master control genes and it is the function of such a gene that is altered upon *tetA*(L) deletion. The experiments here show that the increased expression of *czcD-trkA* and *yheL* is not abolished by restoration of active *tetA*(L). This suggests that there has been a mutation or some kind of irreversible change. If one or more second-site

mutations are in fact involved, the mutation(s) may not obligatorily or immediately accompany *tetA*(L) deletion. Preliminary data (J. Jin and D. H. Bechhofer, unpublished data) indicate that the frequency of *tetA*(L) deletion is just as high as that of a clearly nonessential gene in the same protocol. Thus, TetA(L) functions may not be essential for viability. However, their loss may create selective pressure that leads to the patterns of change that we have begun to characterize here. Further investigation will be required to clarify the timing and nature of the emergence of the irreversible adaptations to *tetA*(L) deletion. It will also be of interest to determine the full panoply of genes that are up-regulated when *tetA*(L) is deleted, e.g., by a DNA array technology. It should be noted that some compensatory adaptations to *tetA*(L) deletion might involve genes that show little or no transcriptional regulation. Even *tetA*(L) itself is predominantly regulated by posttranscriptional mechanisms (27).

The phenotypic type of mutant represented by JC112C presumably exhibits up-regulation of one or more additional genes beyond those genes that are up-regulated in both JC112 and JC112C. In addition to the probable temperature-sensitive Co^{2+} efflux system, JC112C must have an elevated compensatory transport activity(ies) that accounts for its lack of Na^+ sensitivity and only modest alkali sensitivity relative to JC112.

One of the two loci whose expression is markedly elevated in both JC112 and JC112C is the *czcD-trkA* locus. The best-studied homologue, the first *czcD* gene reported, was initially described as having roles in sensing the substrates of the *czcCBA* locus of *R. eutrophus* and in regulating expression of the locus (21, 29). CzcCBA is a toxic divalent cation efflux antiporter whose substrates are cadmium, zinc, and cobalt; the divalent cation is exchanged for external H^+ (22). CzcD is a member of a family of membrane proteins called cation diffusion facilitators (22, 24). Some of these proteins, including CzcD, have most recently been shown to exclude their substrates when expressed in *Ralstonia* (3). The *B. subtilis* CzcD is not as closely related to the *Ralstonia* homologue as many other homologues, including some *B. subtilis* gene products. Paulsen and Saier (24) noted that functions of such less related proteins may not involve or may not solely involve transport of toxic divalent cations. In view of the findings here, it will be of interest to examine the actual transport properties of CzcD and CzcD-TrkA in vitro and, in particular, to determine whether the complex catalyzes divalent cation/K^+ antiport. Such an activity would account for its apparent in vivo contributions to K^+ acquisition and Co^{2+} resistance.

The YheL protein, like its homologue in alkaliphilic *B. firmus* OF4 (15), appears to have a modest role in Na^+-dependent pH homeostasis but makes no detectable contribution to Na^+ resistance. We propose that *yheL* be designated *nhaC*. It is striking that despite the significant up-regulation of *yheL* in both *tetA*(L) deletion mutants, deletion of *yheL* has little effect on these mutants. This is consistent with *yheL* being part of a regulon containing other genes that are more important compensatory genes for TetA(L) functions, e.g., *czcD* and *trkA*. Thus, the strong up-regulation of *yheL* would be a by-product of an effect on the regulon as a whole. In fact, a plausible but tentative hypothesis for the basis of the Co^{2+} sensitivity of JC112 and JC112C (albeit more modest) is an adverse by-product of the elevation of YheL and other proteins. The barrier function of the membrane may be compromised with respect to divalent cation exclusion when one or more of the membrane proteins is elevated as much as YheL is in JC112. The plausibility of this possibility is supported by the marked decrease in the MIC for Co^{2+} in wild-type *B. subtilis* cells transformed with a multicopy plasmid expressing *yheL*. Expression of the gram-negative TetA(C) gene has been correlated with an increase in cadmium sensitivity, and the mechanism is not yet understood (10, 11).

Finally, apart from the enhanced Co^{2+} sensitivity of *tetA*(L) mutants, the demonstrated effect of Co^{2+} on expression of both *czcD-trkA* and *yheL* is notable. It is reasonable to hypothesize that just as Na^+ stress is exacerbated at elevated pH and K^+ insufficiency (23), there may be an intersection among stresses related to TetA(L) function and the stress caused by inhibitory concentrations of toxic divalent cations. For example, Co^{2+} toxicity may be strongly dependent on pH, K^+ status, or Na^+ levels. Any such intersection may be clarified when all genes whose expression is significantly altered in *tetA*(L) deletion mutants have been catalogued.

ACKNOWLEDGMENTS

This work was supported by research grants GM52837 from the National Institute of General Medical Sciences to T.A.K. and from the Inoue Enryo Memorial Foundation for Promoting Science to M.I.

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Amano, H., C. L. Ives, K. F. Bott, and K. Shishido.** 1991. A limited number of *Bacillus subtilis* strains carry a tetracycline-resistance determinant at a site close to the origin of replication. Biochim. Biophys. Acta **1088:**251–258.
3. **Anton, A., C. Grosse, J. Reissmann, T. Pribyl, and D. H. Nies.** 1999. CzcD is a heavy metal ion transporter involved in regulation of heavy metal resistance in *Ralstonia* sp. strain CH34. J. Bacteriol. **181:**6876–6881.
4. **Bechhofer, D. H., and S. J. Stasinopoulos.** 1998. *tetA*(L) mutants of a tetracycline-sensitive strain of *Bacillus subtilis* with the polynucleotide phosphorylase gene deleted. J. Bacteriol. **180:**3470–3473.
5. **Bechhofer, D. H., and W. Wang.** 1998. Decay of *ermC* mRNA in a polynucleotide phosphorylase mutant of *Bacillus subtilis*. J. Bacteriol. **180:**5968–5977.
6. **DiMari, J. F., and D. H. Bechhofer.** 1993. Initiation of mRNA decay in *Bacillus subtilis*. Mol. Microbiol. **7:**705–717.
7. **Cheng, J., A. A. Guffanti, and T. A. Krulwich.** 1994. The chromosomal tetracycline resistance locus of *Bacillus subtilis* encodes a Na^+/H^+ antiporter that is physiologically important at elevated pH. J. Biol. Chem. **269:**27365–27371.
8. **Cheng, J., A. A. Guffanti, W. Wang, T. A. Krulwich, and D. H. Bechhofer.** 1996. Chromosomal *tetA*(L) gene of *Bacillus subtilis*: regulation of expression and physiology of a *tetA*(L) deletion strain. J. Bacteriol. **178:**2853–2860.
9. **Cheng, J., D. B. Hicks, and T. A. Krulwich.** 1996. The purified *Bacillus subtilis* tetracycline efflux protein TetA(L) reconstitutes both tetracycline-cobalt/H^+ and Na^+/H^+ exchange. Proc. Natl. Acad. Sci. USA **93:**14446–14451.
10. **Griffith, J. K., J. M. Buckingham, J. L. Hanners, C. E. Hildebrand, and R. A. Walters.** 1982. Plasmid-conferred tetracycline resistance confers collateral cadmium sensitivity to *E. coli* cells. Plasmid **8:**86–88.
11. **Griffith, J. K., D. H. Cuellar, C. A. Fordyce, K. C. Hutchings, and A. A. Mondragon.** 1994. Structure and function of the class C tetracycline/H^+ antiporter: three independent groups of phenotypes are conferred by TetA(C). Mol. Membr. Biol. **11:**271–277.
12. **Guffanti, A. A., and T. A. Krulwich.** 1995. Tetracycline/H^+ antiport and Na^+/H^+ antiport catalyzed by the *Bacillus subtilis* TetA(L) transporter expressed in *Escherichia coli*. J. Bacteriol. **177:**4557–4561.
13. **Guffanti, A. A., J. Cheng, and T. A. Krulwich.** 1998. Electrogenic antiport activities of the gram-positive Tet proteins include a $Na^+(K^+)/K^+$ mode that mediates net K^+ uptake. J. Biol. Chem. **273:**26447–26454.
14. **Ito, M., A. A. Guffanti, B. Oudega, and T. A. Krulwich.** 1999. *mrpA*: a multigene, multifunctional locus in *Bacillus subtilis* with roles in resistance to cholate and to Na^+, and in pH homeostasis. J. Bacteriol. **181:**2394–2402.
15. **Ito, M., A. A. Guffanti, J. Zemsky, D. M. Ivey, and T. A. Krulwich.** 1997. Role of the *nhaC*-encoded Na^+/H^+ antiporter of alkaliphilic *Bacillus firmus* OF4. J. Bacteriol. **179:**3851–3857.
16. **Ives, C. L., and K. F. Bott.** 1989. Cloned *Bacillus subtilis* chromosomal DNA mediates tetracycline resistance when present in multiple copies. J. Bacteriol. **171:**1801–1810.
17. **Ivey, D. M., A. A. Guffanti, J. S. Bossewitch, E. Padan, and T. A. Krulwich.** 1991. Molecular cloning and sequencing of a gene from alkaliphilic *Bacillus firmus* OF4 that functionally complements an *Escherichia coli* strain carrying a deletion in the *nhaA* Na^+/H^+ antiporter gene. J. Biol. Chem. **266:**23483–23489.
18. **Kosono, S., S. Morotomi, M. Kitada, and T. Kudo.** 1999. Analyses of a *Bacillus subtilis* homologue of the Na^+/H^+ antiporter gene which is impor-

tant for pH homeostasis of alkaliphilic *Bacillus* sp. C-125. Biochim. Biophys. Acta **1409:**171–175.
19. **Krulwich, T. A., J. Cheng, and A. A. Guffanti.** 1994. The role of monovalent cation/proton antiporters in Na^+-resistance and pH homeostasis in *Bacillus*: an alkaliphile *versus* a neutralophile. J. Exp. Biol. **196:**457–470.
20. **Nakamura, T., R. Yuda, T. Unemoto, and E. Bakker.** 1998. KtrAB, a new type of bacterial K^+ uptake system from *Vibrio alginolyticus*. J. Bacteriol. **180:** 3491–3494.
21. **Nies, D. H.** 1992. CzcR and CzcD, gene products affecting regulation of resistance to cobalt, zinc, and cadmium (*czc* system) in *Alcaligenes eutrophus*. J. Bacteriol. **174:**8102–8110.
22. **Nies, D. H., and S. Silver.** 1995. Ion efflux systems involved in bacterial metal resistances. J. Ind. Microbiol. **14:**186–199.
23. **Padan, E., and T. A. Krulwich.** 2000. Sodium stress, p. 117–130. *In* G. Storz and R. Hengge-Aronis (ed.), Bacterial stress responses. ASM Press, Washington, D.C.
24. **Paulsen, I. T., and M. H. Saier, Jr.** 1997. A novel family of ubiquitous heavy metal ion transport proteins. J. Membr. Biol. **156:**99–103.
25. **Shimotsu, H., and D. J. Henner.** 1986. Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. Gene **43:**85–94.
26. **Spizizen, J., and L. Prestidge.** 1969. Conditions for competence in the *Bacillus licheniformis* transformation system. J. Bacteriol. **99:**70–77.
27. **Stasinopoulos, S. J., G. A. Farr, and D. H. Bechhofer.** 1998. *Bacillus subtilis tetA*(L) gene expression: evidence for regulation by translational reinitiation. Mol. Microbiol. **30:**923–932.
28. **Sturr, M. G., A. J. Ablooglu, and T. A. Krulwich.** 1997. A *Bacillus subtilis* locus encoding several gene products affecting transport of cations. Gene **188:**91–94.
29. **Van der Leslie, D., T. Scwuchhow, U. Schwidestzky, S. Wuertz, W. Baeyens, M. Mergeay, and D. H. Nies.** 1997. Two-component regulatory system involved in transcriptional control of heavy-metal homeostasis in *Alcaligenes eutrophus*. Mol. Microbiol. **23:**493–503.
30. **Wang, W., and D. H. Bechhofer.** 1996. Properties of a *Bacillus subtilis* polynucleotide phosphorylase deletion strain. J. Bacteriol. **178:**2375–2382.
31. **Waser, M., D. Hess-Bienz, K. Davies, and M. Solioz.** 1992. Cloning and disruption of a putative Na/H-antiporter gene of *Enterococcus hirae*. J. Biol. Chem. **267:**5396–5400.
32. **Yamaguchi, A., T. Udagawa, and T. Sawai.** 1990. Transport of divalent cations with tetracycline as mediated by the transposon Tn*10*-encoded tetracycline resistance protein. J. Biol. Chem. **265:**4809–4813.