Adaptive Gene Expression in *Bacillus subtilis* Strains Deleted for *tetL*

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The growth properties of a new panel of *Bacillus subtilis* *tetL* deletion strains and of a derivative set of strains in which *tetL* is restored to the chromosome support earlier indications that deletion of *tetL* results in a range of phenotypes that are unrelated to tetracycline resistance. These phenotypes were not reversed by restoration of a *tetL* gene to its native locus and were hypothesized to result from secondary mutations that arise when multifunctional *tetL* is deleted. Such genetic changes would temper the alkali sensitivity and Na^+ sensitivity that accompany loss of the monovalent cation/proton activity of TetL. Microarray comparisons of the transcriptomes of wild-type *B. subtilis*, a *tetL* deletion strain, and its *tetL*-restored derivative showed that 37 up-regulated genes and 13 down-regulated genes in the deletion strain did not change back to wild-type expression patterns after *tetL* was returned to the chromosome. Up-regulation of the *citM* gene, which encodes a divalent metal ion-coupled citrate transporter, was shown to account for the Co^{2+} -sensitive phenotype of *tetL* mutants. The changes in expression of *citM* and genes encoding other ion-coupled solute transporters appear to be adaptive to loss of TetL functions in alkali and Na^+ tolerance, because they reduce Na^+ -coupled solute uptake and enhance solute uptake that is coupled to H^+ entry.

The *tetL* gene of *Bacillus subtilis*, originally identified in a screen of chromosomal mutations that conferred elevated resistance to tetracycline (Tc) (55), was also identified in a screen of transposon insertion mutants that were sensitive to growth in the presence of alkaline pH and elevated Na^+ (7). Studies on the functions of *tetL* have since implicated this gene in several key areas of cell physiology. It has been shown that TetL plays important roles in alkaline pH homeostasis, Na^+ resistance, and acquisition of K^+ , in addition to its role in Tc resistance (8, 35, 36, 51). The basis for the multiple physiological roles are the multiple catalytic capacities of the TetL antiporter. TetL supports antibiotic resistance by an efflux mechanism in which a Tc-divalent metal complex is exchanged for a greater number of external protons, i.e., electrogenic $[\text{Tc-Me}]^+/\text{H}^+$ antiport (20, 21, 39); neither Tc nor the divalent cation can serve as an efflux substrate without the other (9, 20, 39, 56). The pH homeostasis and Na^+ resistance functions of TetL depend upon its additional capacity for Na^+ (K^+)/ H^+ antiport (8) and K^+ acquisition modes. TetL-mediated K^+ uptake occurs when K^+ substitutes for some of the external H^+ coupling ions in a competitive manner that depends upon their relative concentrations in the medium and their electrochemical potentials (21, 29).

The studies that established the physiological roles for TetL included the construction of strains that were deleted for the *tetL* locus, the properties of which indicated that TetL is a major Na^+ (K^+)/ H^+ antiporter required for pH homeostasis (8). Deletion of *tetL* results in strains that show varied phenotypes. One type is exemplified by JC112, a strain that is defective for growth at both neutral and alkaline pH and in the presence of limiting K^+ and that is also defective in alkaline pH homeostasis and Na^+ extrusion (8). These properties are

consistent with the role of TetL as a major Na^+/H^+ antiporter. JC112 also grows poorly in the presence of Co^{2+} , a property that was suggested to relate to up-regulation of other membrane proteins in response to the absence of TetL (51). Another type of *tetL* deletion mutant, which is the more prevalent isolate, is represented by JC112C, a strain that shows a more moderate phenotype than JC112 with respect to the above-mentioned properties (51).

A selected group of genes, predicted to encode various types of antiporters, were screened for increased expression in JC112 and JC112C. Two antiporters that showed elevated expression levels were *nhaC*, a Na^+/H^+ antiporter gene, and *czcD-czcO*, a divalent metal resistance locus. The increase in *czcD-czcO* mRNA in Northern analyses was especially pronounced when Co^{2+} was added to the medium, and neither this increase nor the increase in basal expression was altered by the presence of a wild-type *tetL* gene in the *amyE* locus (51). These studies led to the hypothesis that the ability of *B. subtilis* to compensate for deletion of *tetL* requires secondary-site mutations. The current studies were undertaken to test this hypothesis and to better understand the response of *B. subtilis* to the loss of *tetL*. For this purpose, a mutant with a substitution of the *tetL* coding sequence was constructed, the *tetL* gene was restored at the native locus in several deletion mutants, and the transcriptomes of a *tetL* deletion strain and its derivative *tetL*-restored strain were assessed for changes in gene expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* parent strain was BD99, which is *hisA1 thr-5 trpC2*. The preparation and transformation of *B. subtilis* competent cell cultures were as described previously (13). Transformed strains were selected on solid Luria-Bertani medium with 80 mM K^+ replacing Na^+ (LBK) (18), using 4 $\mu\text{g}/\text{ml}$ chloramphenicol (Cm), 100 $\mu\text{g}/\text{ml}$ spectinomycin (Sp), 3 $\mu\text{g}/\text{ml}$ phleomycin (Pm), or 5 $\mu\text{g}/\text{ml}$ erythromycin (Em). *Escherichia coli* strains DH5 α (19) and XL1Blue (Stratagene) were the hosts for plasmid constructions.

B. subtilis strains were grown at 30°C. TTM medium was used for assays of Tc resistance to minimize competitive inhibition by the high potassium ion content

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FIG. 1. Schematic diagram of the *tetL* locus. The middle line depicts the *tetL* locus, indicating the *tetL* leader peptide CDS (hatched box) and *tetL* CDS (open box). The promoter (P) and terminator (t) are indicated. Relevant restriction sites are shown. The top line represents replacement of the *tetL* locus with the *cat* cassette in JC112 and JC112C. The bottom line represents replacement of the *tetL* leader and *tetL* CDS with a *cat* CDS in strains JC203, JC205, and JC206. The location and direction of transcription of genes upstream and downstream of *tetL* are indicated, as well as the site of insertion of an *Sp* resistance gene in the upstream *yyaM* gene.

of other media (29). TTM consists of 100 mM Tris-HCl buffer (pH 7.0 or 8.3); 1 mM potassium phosphate; 0.01% MgSO_4 ; 0.2% $(\text{NH}_4)_2\text{SO}_4$; 50 μg (each) of L-threonine, L-histidine, and L-tryptophan per ml; 0.1% yeast extract; and 50 mM Tris malate. The medium was adjusted to pH 7.0 for assays of Tc resistance. For TKM medium, potassium malate replaced the Tris malate. Pm resistance was assayed in LBK medium at pH 7.5. For all other experiments, including isolation of RNA for microarray analyses, cells were grown in Spiz-KM medium, consisting of 44 mM KH_2PO_4 , 80 mM K_2HPO_4 , 15 mM $(\text{NH}_4)_2\text{SO}_4$, 3.4 mM sodium citrate, 50 mM potassium malate, 0.1 mM MgSO_4 , 0.01% yeast extract, and 50 $\mu\text{g}/\text{ml}$ each of histidine, threonine, and tryptophan. Where specified, potassium citrate replaced sodium citrate in this medium. The pH of the medium is indicated for individual experiments.

Plasmid constructions. To replace the *tetL* leader peptide coding sequence (CDS) and *tetL* CDS with a Cm resistance gene (*cat*) CDS, plasmid pYW112 was used. The complete *cat* CDS from plasmid pC194 (26) was amplified by PCR as an *NdeI*-*DraI* fragment; the *NdeI* restriction site (CATATG) contained the ATG start codon, and the *DraI* restriction site (TTTAAA) contained the TAA stop codon. Two other PCR fragments were prepared; one was a 1,140-bp fragment containing sequences upstream of *tetL* on a *BstBI*-*NdeI* fragment, and the other was a 570-bp fragment containing sequences downstream of *tetL* on a *DraI*-*HindIII* fragment. The three PCR fragments were ligated into the vector plasmid pYH56 (10), between *Clal* and *HindIII* sites, giving plasmid pYW112. Plasmid pYW112 was used to transform BD99, with selection for Cm resistance. Recombination by double crossover resulted in a clean replacement of the leader peptide CDS and *TetL* CDS with a *cat* CDS, whose expression is controlled by the *tetL* promoter and transcription terminator and the *tetL* leader peptide translational signals. This and all other plasmid constructs were confirmed by DNA sequencing.

To restore *tetL* in the deletion mutants, *tetL*-deleted strains were transformed with genomic DNA from BG384, a *B. subtilis* *thr-5* *trpC2* strain that has a *Sp* resistance cassette inserted in the *MunI* site upstream of *tetL*, as shown in Fig. 1 (1).

To restore *tetL* to its original chromosomal site without introducing an antibiotic cassette near the *tetL* locus, an intermediate plasmid, named pYW124, was constructed. A 4.5-kilobase chromosomal *HindIII* fragment containing the *tetL* gene (7) was amplified in two parts, with the internal primers providing a *Bam*HI site by an A-to-T change 180 bp downstream of the *tetL* stop codon. The two fragments were cloned into pGEM-9Zf(+) (Promega), yielding plasmid pYW124. The *HindIII* insert was then transferred to the temperature-sensitive plasmid pG⁺host4 (37), to give plasmid pYW124G. pYW124G was used to transform BD99, JC112, or JC210, with selection for Em resistance at 30°C. Colonies with integration of pYW124G were selected on Em plates at 42°C. Integration at the *tetL* locus was confirmed by PCR. Colonies with integrated pYW124G were grown at 30°C without selection and were spread for isolated colonies. Colonies that had undergone a double crossover to restore *tetL* were screened by Em sensitivity and, in the case of JC112, Tc resistance, and the desired construct was confirmed by *Bam*HI digestion of the PCR-amplified *tetL* locus. Southern blot analysis was done to confirm that the *tetL* locus was restored and that there was no amplification of the *tetL* gene.

For *citM* knockout strain construction, plasmid pYW119 was used. This plasmid contained the following three PCR amplification products: a 510-bp *Bam*HI-XbaI fragment that contained sequences from upstream of the *citM* gene to bp 45 of the *citM* CDS, an 850-bp XbaI-KpnI fragment that contained the Pm resistance gene of plasmid pUB110 (45), and a 570-bp KpnI-EcoRI fragment that contained the last 60 bp of the *citM* CDS followed by downstream sequence. The three PCR products were ligated together between the *Bam*HI and *Eco*RI sites of pGEM3-Zf(+).

B. subtilis strains used in the study are listed in Table 1.

RNA analysis. RNA isolation and Northern blot analysis were done exactly as described previously (11), except that the cells were grown in Spiz-KM at pH 7.0. Uniformly labeled, antisense riboprobes were synthesized by T7 RNA polymerase transcription, in the presence of [α - ^{32}P]UTP, of gel-purified DNA fragments that were generated by PCR amplification of the various coding sequences. The upstream primer in the PCRs contained the 17-nucleotide T7 RNA polymerase promoter sequence at its 5' end. Probing of 5S RNA for quantitation of Northern blots was done using a 5'-end-labeled oligonucleotide.

The microarray experiments were done as described previously (11). Briefly, total RNA was extracted by hot-phenol extraction, using a buffer containing 50 mM sodium acetate and 1 mM EDTA (pH 5.1). cDNA was synthesized in the presence of cyanine 3-dCTP (Cy3) and cyanine 5-dCTP (Cy5) dyes (Perkin-Elmer Life Sciences). The Cy3-cDNA (from the reference strain BD99 or JC112) and Cy5-cDNA (from JC112 or JC112-R) probes were concentrated and mixed. The mixture was hybridized to *B. subtilis* microarray slides (Eurogentec) overnight at 42°C in an Amersham Lucidea Slidepro hybridization station. The microarray slides contained whole open reading frame sequences for 4,096 of the 4,106 *B. subtilis* genes, and each *B. subtilis* gene was represented twice on the microarray. After washing, the slides were scanned at excitation wavelengths of 550 nm (Cy3) and 640 nm (Cy5) in a ScanArray ExpressHT scanner. Fluorescence was measured at 570 nm (Cy3) and 670 nm (Cy5). The fluorescence intensity of microarray spots was quantified and analyzed using the ScanArray Express program. A normalized Cy5/Cy3 mean fluorescence ratio was used to quantify changes in gene expression.

TABLE 1. *B. subtilis* strains used in this study

Strain	Relevant characteristics
BD99	 <i>hisA1 thr-5 trpC2</i> ; "wild type"
BD99-05	BD99 with Cm resistance cassette in <i>amyE</i> locus
BD99 Δ <i>citM</i>	BD99 with Pm resistance cassette gene replacing <i>citM</i>
JC112	BD99 with Cm resistance cassette replacing <i>tetL</i>
JC112-R	JC112 with restored <i>tetL</i> gene at native locus
JC112 Δ <i>citM</i>	JC112 with Pm resistance cassette gene replacing <i>citM</i>
JC112C	BD99 with Cm resistance cassette replacing <i>tetL</i>
JC112C-R	JC112C with restored <i>tetL</i> gene at native locus
JC203	BD99 with Cm resistance CDS substitution of <i>tetL</i> CDS
JC203-R	JC203 with restored <i>tetL</i> gene at native locus
JC205	BD99 with Cm resistance CDS substitution of <i>tetL</i> CDS
JC205-R	JC205 with restored <i>tetL</i> gene at native locus
JC206	BD99 with Cm resistance CDS substitution of <i>tetL</i> CDS
JC206-R	JC206 with restored <i>tetL</i> gene at native locus
JC210	BD99 with <i>Sp</i> resistance cassette inserted in <i>yyaM</i> locus
BG519	BD99 <i>rocR</i>
BG520	JC112 <i>rocR</i>

TABLE 2. Overnight growth of *tetL* deletion mutants and the corresponding strains with *tetL* restored, relative to that of the wild-type strain

Strain	Tetracycline MIC ($\mu\text{g/ml}$) in TTM ^a	Alkali resistance (%) in pH 8.3 TKM ^b	Na ⁺ resistance (%) in pH 7.0 TKM + 1.2 M NaCl ^c	Co ²⁺ resistance (%) in pH 7.0 Spiz-KM + 300 μM CoCl ₂ ^d
BD99	1.95 $\pm$ 0.24	100.0 $\pm$ 4.93	100.0 $\pm$ 3.02	100.0 $\pm$ 6.86
JC112	0.04 $\pm$ 0.00	54.5 $\pm$ 3.0	77.8 $\pm$ 2.73	12.3 $\pm$ 4.53
JC112C	0.09 $\pm$ 0.01	91.3 $\pm$ 5.63	120.0 $\pm$ 7.16	50.6 $\pm$ 5.00
JC203	0.09 $\pm$ 0.01	76.4 $\pm$ 4.71	78.8 $\pm$ 3.22	72.8 $\pm$ 13.7
JC205	0.09 $\pm$ 0.00	69.5 $\pm$ 4.48	73.6 $\pm$ 4.41	119.2 $\pm$ 17.8
JC206	0.05 $\pm$ 0.00	79.8 $\pm$ 4.48	43.5 $\pm$ 2.65	99.6 $\pm$ 8.73
JC210	2.20 $\pm$ 0.11	107.4 $\pm$ 4.45	124.0 $\pm$ 5.64	108.5 $\pm$ 9.72
JC112-R	1.58 $\pm$ 0.02	55.2 $\pm$ 3.13	78.9 $\pm$ 3.55	6.20 $\pm$ 1.17
JC112C-R	1.95 $\pm$ 0.12	91.8 $\pm$ 6.09	119.3 $\pm$ 6.40	50.0 $\pm$ 4.10
JC203-R	2.18 $\pm$ 0.14	74.8 $\pm$ 4.78	80.5 $\pm$ 4.28	55.4 $\pm$ 11.7
JC205-R	2.46 $\pm$ 0.03	70.0 $\pm$ 3.91	68.5 $\pm$ 4.33	114.4 $\pm$ 12.2
JC206-R	2.28 $\pm$ 0.20	78.3 $\pm$ 3.80	31.1 $\pm$ 2.95	97.1 $\pm$ 10.3

^a Minimal concentration at which growth (A_{600}) was below 0.1 after 16 h at 30°C. Results are averages of four determinations $\pm$ SEM.

^b A_{600} values for growth of BD99 in 16 h at pH 8.3 and pH 7.0 were determined, and the percentage of growth at pH 8.3 relative to growth at pH 7.0 was set at 100%. Growth of other strains at pH 8.3 and pH 7.0 was measured, and the values shown are the ratio of growth of the strain relative to that of BD99. Results are averages from at least four experiments $\pm$ SEM.

^c A_{600} values for growth of BD99 in 16 h at pH 7.0 with and without 1.2 M NaCl were determined, and the percentage of growth in the presence of 1.2 M NaCl relative to growth without 1.2 M NaCl was set at 100%. Growth of other strains with and without 1.2 M NaCl was measured, and the values shown are the ratio of growth of the strain relative to that of BD99. TKM medium has a background NaCl concentration of about 5 mM. Results are averages from at least four experiments $\pm$ SEM.

^d A_{600} values for growth of BD99 in 8 h at pH 7.0 with and without 300 μM CoCl₂ were determined, and the percentage of growth in the presence of CoCl₂ relative to growth without CoCl₂ was set at 100%. Growth of other strains with and without 300 μM CoCl₂ was measured, and the values shown are the ratio of growth of the strain relative to that of BD99. Results are averages from at least four experiments $\pm$ SEM.

Genes with a Cy5/Cy3 mean fluorescence ratio of >3 were considered significantly up-regulated, while genes with a mean fluorescence ratio of <0.33 were considered significantly down-regulated.

Statistical analysis of growth data. Standard errors of the means (SEM) for ratios reported in Table 2 were computed as described by Finney (14).

RESULTS

New *tetL* deletion mutants. *tetL* deletion mutants JC112 and JC112C were constructed previously by transforming wild-type strain BD99 with a plasmid that had a Cm resistance cassette replacing sequences from the ClaI site located 272 bp upstream of the *tetL* CDS to an NdeI site located 159 bp downstream of the *tetL* CDS (Fig. 1). Thus, there was some concern that the phenotypes observed with JC112 and JC112C may be due, at least in part, to effects of the deleted *tetL* locus on expression of neighboring genes. We observed previously that there is a significant amount of readthrough past the *tetL* transcription terminator (8). Therefore, a plasmid that had a precise substitution of only the *tetL* leader peptide and CDS was constructed, using the *cat* CDS from the same element that was used to knock out *tetL* previously (Fig. 1). Control exper-

iments demonstrated that the same Cm cassette in the *amyE* locus did not produce detectable phenotypes other than Cm resistance (strain BD99-05; data not shown). The plasmid was used to transform BD99. Twenty-seven Cm-resistant, Tc-sensitive clones were obtained by selection on LBK plates, as opposed to the more defined Spiz-KM used in the isolation of the original set of *tetL* deletion mutants. Genomic DNA from the new clones was analyzed by PCR to confirm the presence of the coding sequence substitution and by Southern blot analysis to eliminate clones that might have amplifications of the *tetL* locus, as has been observed previously by us and others (1, 28). The clones were grouped into categories based on 15-hour growth in 0.2 $\mu\text{g/ml}$ Tc in semidefined TKM medium (see Materials and Methods). Nine clones were chosen for further analysis, and these were grouped into categories based on 15-hour growth in 1.4 M NaCl in TKM medium (pH 7.0) and 8-hour growth in 400 μM CoCl₂ in Spiz-KM medium (data not shown). Of these, three clones were chosen (JC203, JC205, and JC206) for the following analyses: MICs for growth in Tc in TTM medium, growth in the presence of inhibitory levels of NaCl in TKM medium at pH 7.0 and 8.3, and growth in Spiz-KM medium in the presence of 300 μM CoCl₂ (Table 2). The three clones showed a range of phenotypes, similar to the range found previously when the greater *tetL* locus was deleted (51). For example, JC205 was the most sensitive to alkali conditions but the least sensitive to the presence of Co²⁺. JC206, on the other hand, was the least sensitive to alkali but the most sensitive to Na⁺. None of the three *tetL* deletion mutants had the same phenotypes as the original JC112 and JC112C. These results demonstrated that the absence of TetL protein alone was responsible for defects in growth at high pH and elevated monovalent and divalent cation concentrations. We postulate that loss of *tetL* results in selective pressure that leads to second-site mutations that allow better growth of *tetL*-deleted strains.

Restoration of the native *tetL* locus. If deletion of *tetL* was indeed accompanied by second-site mutations, it was predicted that restoration of *tetL* to a deleted strain would not necessarily complement all of the mutant phenotypes. To examine more precisely the effect of restoring *tetL*, derivatives of the three clones mentioned above, as well as of JC112 and JC112C, were constructed in which the deleted *tetL* locus was restored to wild type. The *tetL*-deleted strains were transformed with chromosomal DNA from a strain that had the wild-type *tetL* locus and a Sp resistance gene inserted at a MunI site located 1,804 bp upstream of the *tetL* CDS (Fig. 1). This site is in the coding sequence of a gene named *yjaM*, which is annotated in the SubtiList database as having an unknown function and being similar to other *B. subtilis* proteins of unknown function; a control wild-type strain with this insertion (JC210; Table 2) was phenotypically similar to the wild type with respect to Tc resistance, Na⁺ sensitivity, and Co²⁺ sensitivity. The presence of wild-type *tetL* in the strains transformed to Sp resistance was confirmed by PCR analysis. Revertants were designated with the addition of an "R" to the strain number.

The data in Table 2 show that the presence of the wild-type *tetL* restored Tc resistance, as previously observed (51). On the other hand, wild-type growth yields under other conditions were not restored and, in some cases, adding back the *tetL* gene actually resulted in lower levels of growth, e.g., growth of

TABLE 3. Transcript analysis of JC112 and JC112-R: genes overexpressed relative to those in BD99

Gene	Operon	Protein description ^a	Fold change in:		Restored to wild type	JC112-R similar to JC112 ^b	JC112-R different from JC112 but not wild type
			JC112	JC112-R			
<i>acsA</i>	<i>citM-yfiN</i>	Acetyl coenzyme A synthetase	6.3	10.4		+	
<i>citM</i>		Secondary transporter of the Mg ²⁺ /citrate complex	5.0	5.4		+	
<i>dhaS</i>	<i>dppA-E</i>	Aldehyde dehydrogenase	3.8	6.9		+	
<i>dppE</i>		Dipeptide ABC transporter (dipeptide-binding protein)	3.6	4.4		+	
<i>gapB</i>	<i>gapBSPED</i>	Glyceraldehyde-3-phosphate dehydrogenase	12.7	14.6		+	
<i>gsiB</i>		General stress protein	6.1	18.8			+
<i>licH</i>	<i>licRBCAH</i>	6-Phospho-beta-glucosidase	5.2	8.3		+	
<i>phrK</i>	<i>rapK-phrK</i>	Regulator of the activity of phosphatase RapK	5.0	5.2		+	
<i>sdhB</i>	<i>sdhCAB</i>	Succinate dehydrogenase (iron-sulfur protein)	3.6	2.3		+	
<i>ydaG</i>		Sigma-B-dependent; similar to general stress protein	6.0	15.9			+
<i>ykzA</i>		Sigma-B-dependent; similar to general stress protein	3.8	10.2			+
<i>yomL</i>	<i>yomL-yozP</i>	Unknown	5.8	10.3		+	
<i>ysfD</i>	<i>ysfBCD</i>	Similar to glycolate oxidase subunit	24.2	23.3		+	
<i>ytdI</i>		Similar to unknown proteins	5.3	7.2		+	
<i>ywfM</i>		Similar to unknown proteins	5.7	6		+	
<i>acoA</i>	<i>acoABCL</i>	Acetoin dehydrogenase E1 component (TPP-dependent alpha subunit)	13.3	23.5		+	
<i>acoB</i>		Acetoin dehydrogenase E1 component (TPP-dependent beta subunit)	17.7	24.2		+	
<i>acoC</i>		Acetoin dehydrogenase E2 component (dihydroliipoamide acetyltransferase)	34.9	59.7		+	
<i>acoL</i>		Acetoin dehydrogenase E3 component (dihydroliipoamide dehydrogenase)	35.6	60.5		+	
<i>acoR</i>	3' to <i>acoABCL</i>	Positive regulation of the acetoin dehydrogenase operon	9.5	12.5		+	
<i>rbsR</i>	<i>rbsRKDACB</i>	Negative regulation of the ribose operon	3.4	3.6		+	
<i>rbsK</i>		Ribokinase	5.0	5.3		+	
<i>rbsD</i>		Ribose ABC transporter (membrane protein)	8.6	7.5		+	
<i>rbsA</i>		Ribose ABC transporter (ATP-binding protein)	13.3	17.7		+	
<i>rbsC</i>		Ribose ABC transporter (permease)	6.0	10.3		+	
<i>rbsB</i>		Ribose ABC transporter (ribose-binding protein)	15.4	24.4		+	
<i>rocG</i>	<i>rocGABC</i>	Glutamate dehydrogenase	10.7	10.5		+	
<i>rocA</i>		Pyrroline-5 carboxylate dehydrogenase	13.9	11.7		+	
<i>rocB</i>		Involved in arginine and ornithine utilization	7.3	9.5		+	
<i>rocC</i>		Amino acid permease	4.4	3.4		+	
<i>rocD</i>	<i>rocDEF</i>	Ornithine aminotransferase	9.5	7.6		+	
<i>rocE</i>		Amino acid permease	10.4	7.2		+	
<i>rocF</i>		Arginase	8.3	7.5		+	
<i>yvdF</i>	<i>yvdE-K-malL-pgcM</i>	Similar to glucan 1,4-alpha-maltohydrolase	6.3	3	+		
<i>yvdG</i>		Similar to maltose/maltodextrin-binding protein	4.3	3.3		+	
<i>malL</i>		Oligo-1,4-1,6-alpha-glucosidase (sucrase-maltase-isomaltase)	6.2	4.3		+	
<i>pgcM</i>		Beta-phosphoglucomutase and glucose-1-phosphate phosphodismutase	4.2	2.5		+	

^a Function or comment in SubtiList database.^b Less than a twofold difference between JC112 and JC112-R is considered similar.

JC206R in the presence of elevated sodium, and growth of JC203R in the presence of Co²⁺. For strains JC112 and JC112C, there were no significant effects of *tetL* restoration on growth in the various conditions.

Similar conclusions were drawn from a set of strains in which *tetL* was restored without introducing an antibiotic resistance cassette in the *tetL* locus. A marked copy of the *tetL* gene

containing a single-base-pair change located 180 bp downstream of the *tetL* CDS, which created a BamHI site, was introduced into the chromosome of JC112 (see Materials and Methods). This version of the *tetL*-restored JC112, which, unlike JC112-R, did not contain an inserted Sp resistance cassette, also did not show restoration of the *tetL* deletion phenotypes (data not shown).

TABLE 4. Transcript analysis of JC112 and JC112R: genes underexpressed relative to those in BD99

Gene	Operon	Protein description ^a	Fold change in:		Restored to wild type	JC112-R similar to JC112 ^b	JC112-R different from JC112 but not wild type
			JC112	JC112-R			
<i>gltA</i>	<i>gltAB</i>	Glutamate synthase (large subunit)	4.3	12.5			+
<i>maeN</i>		Na ⁺ /malate symporter	25	33.3		+	
<i>ycsF</i>	<i>ycsFGI-kipLAR-ycsK</i>	Expression activated by TnrA and repressed by KipR; similar to lactam utilization protein	4.5	7.7		+	
<i>ysbA</i>	<i>thrS-lytST-ysbAB</i>	Similar to hypothetical proteins from <i>B. subtilis</i>	6.9	15.4			+
<i>ywkA</i>	<i>ywkAB</i>	Similar to malate dehydrogenase	8	10.3		+	
<i>dhbC</i>	<i>dhbACEBF</i>	Isochorismate synthase	4.8	3.6		+	
<i>dhbB</i>	<i>dhbACEBF</i>	Isochorismatase	4	3		+	
<i>nrgA</i>	<i>nrgAB</i>	Ammonium transporter	16.7	22.2		+	
<i>nrgB</i>	<i>nrgAB</i>	Nitrogen-regulated PII-like protein	3.8	3.9		+	
<i>treP</i>	<i>trePAR</i>	Phosphotransferase system trehalose-specific enzyme IIBC component	14.8	12.9		+	
<i>treR</i>	<i>trePAR</i>	TreR transcriptional regulator (GntR family)	6.2	6.2		+	
<i>xtmA</i>	<i>xtmA-xtmB-xkdABEFGHIJKM</i>	PBSX defective prophage terminase small subunit	4.1	1.7	+		
<i>xkdF</i>	<i>xtmA-xtmB-xkdABEFGHIJKM</i>	PBSX prophage gene; similar to YqbD of the skin element	7.3	1.9	+		
<i>xkdG</i>	<i>xtmA-xtmB-xkdABEFGHIJKM</i>	PBSX prophage gene; similar to YqbE of the skin element	6.9	2.5	+		
<i>xkdH</i>	<i>xtmA-xtmB-xkdABEFGHIJKM</i>	PBSX prophage gene; similar to YqbH of the skin element	6.2	2.3	+		
<i>xkdI</i>	<i>xtmA-xtmB-xkdABEFGHIJKM</i>	PBSX prophage gene; similar to YqbI of the skin element	4	2.9		+	
<i>xkdJ</i>	<i>xtmA-xtmB-xkdABEFGHIJKM</i>	PBSX prophage gene; similar to YqbJ of the skin element	7	2.5	+		
<i>xkdK</i>	<i>xtmA-xtmB-xkdABEFGHIJKM</i>	PBSX prophage gene	7.7	3.7			
<i>xkdM</i>	<i>xtmA-xtmB-xkdABEFGHIJKM</i>	PBSX prophage gene; similar to YqbM of the skin element	7.1	2.1	+		
<i>xkdO</i>	<i>xkdN-Z-xepA-xhLAB-xlyA</i>	PBSX prophage gene; similar to YqbO of the skin element	4.4	1.8	+		
<i>xkdP</i>	<i>xkdN-Z-xepA-xhLAB-xlyA</i>	PBSX prophage gene; similar to YqbP of the skin element	5.4	2.7	+		
<i>xkdQ</i>	<i>xkdN-Z-xepA-xhLAB-xlyA</i>	PBSX prophage gene; similar to YqbQ of the skin element	4.1	3.3		+	
<i>xepA</i>	<i>xkdN-Z-xepA-xhLAB-xlyA</i>	Lytic exoenzyme associated with defective prophage PBSX	3.5	2.2		+	
<i>xlyA</i>	<i>xkdN-Z-xepA-xhLAB-xlyA</i>	N-Acetylmuramoyl-L-alanine amidase	4.3	1.5	+		
<i>ykuN</i>	<i>ykuNOPQ</i>	Similar to flavodoxin	5.2	3.6		+	
<i>ykuP</i>	<i>ykuNOPQ</i>	Similar to sulfite reductase	5.1	3.4		+	

^a Function or comment in SubtiList database.^b Less than a twofold difference between JC112 and JC112-R is considered similar.

The remaining studies focused on JC112, the best characterized of the *tetL* deletion mutants.

Transcriptome analysis: overexpressed genes. Microarray experiments were performed to assess global changes in gene expression in JC112 and the effect of restoring *tetL* in JC112-R. mRNA levels in these two strains were compared to those in BD99. The data in Tables 3 and 4 are averages from duplicate samples in two independent experiments (i.e., averages of four measurements). Shown are changes that were more than three-

fold, which was considered significant. Five genes, which encoded products that could be related to the alkali, Na⁺, or Co²⁺ sensitivity of the deletion strain, were selected for Northern blot analysis, to confirm the microarray findings (Fig. 2).

The data for 37 genes that had a positive fold change (i.e., overexpression) (Table 3) can be summarized as follows. Genes in the *rocG* and *rocD* operons, required for arginine metabolism (15), were overexpressed in JC112. Northern blot analysis of the *rocD* operon, using a *rocD* riboprobe, gave

FIG. 2. Northern blot analysis of selected transcripts, as indicated on the left, from the strains indicated above the lanes. The fold change in each strain, relative to BD99, is shown below each lane. Data are averages from two experiments.

results similar to those with the microarray (Fig. 2). The Northern blot analysis further showed that the increased level of *rocD* mRNA was abolished upon deletion of *rocR*, the positive regulator of the *roc* operons (6), from JC112 (strain BG520) (Fig. 2). We postulated that increased arginine uptake and degradation to glutamate would provide a means of acidifying the cytoplasm and/or the medium in the absence of the major proton uptake activity provided by *tetL*. Additionally, the up-regulated arginine (ornithine) transporters RocE and RocC probably import the amino acid together with H^+ rather than with Na^+ , since they are related to H^+ -coupled LysP from *E. coli* rather than to families of Na^+ -coupled amino acid transporters (www.membranetransport.org).

Genes in three operons involved in carbohydrate metabolism—the *aco*, *rbs*, and *yvd* operons—were overexpressed in JC112. How this relates to deletion of *tetL* was not clear, and this was not addressed in the current study.

Overexpressed monocistronic genes, or cases where only one gene in an operon was overexpressed, are listed in the top part of Table 3. As could be expected, several stress protein-encoding genes were included, as well as other genes involved in carbohydrate metabolism. Of particular interest was the fivefold increase in *citM* gene expression, since *citM* encodes a divalent cation/citrate symporter (31, 34, 52). The increase in *citM* mRNA was confirmed by Northern blot analysis (Fig. 2).

In the comparison of changes in gene expression between JC112 and JC112-R, 33 out of 37 genes had similar levels of expression (we arbitrarily chose a less than twofold change between JC112 and JC112-R as being similar). The restoration of wild-type *tetL* resulted in virtually none of the 37 genes being expressed at the wild-type level (the one case of *yvdF* is likely unreliable, as other genes in the same operon showed no change according to our arbitrary definition and the significance of a threefold change was borderline). In fact, the only notable change between JC112 and JC112-R was the approximately threefold higher expression of three genes (*gsiB*, *ydaG*, and *ykzA*) encoding stress proteins.

Transcriptome analysis: underexpressed genes. The data for 26 genes that had a negative fold change (i.e., underexpression) (Table 4) can be summarized as follows.

Half of the underexpressed genes in JC112 were in operons of the PBSX prophage, and these were generally reverted to wild-type levels in JC112-R. Since none of the overexpressed genes had returned to wild-type levels in JC112-R, this result was important in demonstrating that restoration of *tetL* could indeed reestablish altered gene expression in some cases.

The remaining half of the down-regulated genes included the *maeN* gene, which encodes a Na^+ /malate symporter (53); *maeN* was expressed at very low levels in JC112 and JC112-R relative to the wild type. This was confirmed by Northern blot analysis, which also showed that the *rocR* status of JC112 did not affect the level of *maeN* expression (Fig. 2). The rationale for a mutation resulting in such a change seemed straightforward: import of Na^+ would need to be avoided in the absence of TetL, the major contributor to alkaline pH homeostasis and a contributor to Na^+ resistance. Since the uptake of malate by *maeN* is coupled to uptake of Na^+ , the cell would benefit from reducing this mode of using malate as a carbon source.

After *maeN*, the gene that was most highly repressed in JC112 as well as JC112-R was the *nrgA* gene, which encodes an ammonium transporter (12). Northern blot analysis of *nrgA* mRNA also showed down-regulation (Fig. 2), although not as dramatic as that suggested by the microarray data. We postulated that the down-regulation of *nrgA* in JC112 might be linked to *roc* operon overexpression, since *roc*-mediated arginine utilization would generate sufficient internal NH_4^+ to eliminate the requirement for additional NH_4^+ import via *nrgA*. The observed down-regulation of *gltA* in JC112 and JC112-R was also expected in the context of overexpressed *roc* operons, for two reasons: first, conversion of arginine to glutamate via the *roc* pathway would obviate the need for glutamate synthesis by *gltAB*, and second, *gltA* expression is positively regulated by *gltC*, and expression of *gltC* is in turn negatively regulated by a *rocG*-dependent pathway (3). Thus, the 10-fold increase in *rocG* expression in JC112 would result in repression of *gltC*, which is needed for up-regulation of *gltA*. To test these hypotheses with respect to *nrgA* and *gltA* down-regulation in JC112, Northern blot analysis of *nrgA* and *gltA* expression was performed on BD99, JC112, and their derivative *rocR*-deleted strains, BG519 and BG520. As shown in Fig. 2, Northern blot analysis of *gltA* expression in the *roc*-deleted strains showed that *gltA* expression was restored as expected almost to the wild-type level in BG520, the JC112 strain that had *rocR* deleted. However, the results did not support the idea that elevated *roc* operon expression is the cause of decreased *nrgA* mRNA, since *nrgA* expression was down even in the *rocR*-deleted strains (Fig. 2).

(Note that the *tetL* gene itself is not included in Table 4, although it is present in the wild type and absent in JC112, because the microarray slides we used do not contain the complete set of *B. subtilis* genes. About 10 open reading frames are not represented, including *tetL*.)

Additional data (Table 5) include genes that are unchanged in JC112 but that have higher or lower levels of expression in JC112-R. These were not investigated further.

Alkalinization of growth medium. As another test of the effect of *tetL* deletion and restoration, measurements of the pH

TABLE 5. Changes in transcript levels in JC112-R but not in JC112

Gene	Operon	Protein description ^a	Fold change in transcript level in JC112-R
Upregulated genes			
<i>ycbP</i>		Similar to unknown proteins from <i>B. subtilis</i>	8.1
<i>ycdF</i>	<i>ycdFG</i>	Sigma B-dependent; similar to glucose 1-dehydrogenase	6
<i>ydaD</i>	<i>ydaDEFG</i>	Sigma B-dependent; similar to alcohol dehydrogenase	4.5
<i>ydaS</i>	<i>ydaTS</i>	Unknown	5.7
<i>ydbD</i>		Similar to manganese-containing catalase	9
<i>yflN</i>	<i>citM yflN</i>	Positively regulated by CitST (citrate) and negatively regulated by CcpA (glucose); similar to unknown proteins	4.9
<i>ygbB</i>		Unknown	6.8
<i>yhlL</i>	<i>yhlLKJ</i>	Similar to sensory transduction pleiotropic regulatory protein	12.2
<i>yjbD</i>	<i>yjbBCD</i>	Similar to unknown proteins	4.4
<i>yvaA</i>		Similar to unknown proteins	6.1
<i>ywtG</i>		Similar to metabolite transport protein	7.1
<i>ywzA</i>		Similar to unknown proteins from <i>B. subtilis</i>	8.5
<i>ytxG</i>	<i>ytxGHJ</i>	General stress regulon controlled by sigma B; induced by salt addition during logarithmic growth	5.4
<i>ytxJ</i>	<i>ytxGHJ</i>	General stress regulon controlled by sigma B; induced by salt addition during logarithmic growth	5.9
Downregulated genes			
<i>glnR</i>	<i>ynbAB-glnRA</i>	Transcriptional regulator	4.4
<i>glbB</i>	<i>glbAB</i>	Glutamate synthase (small subunit)	6.9
<i>infC</i>	<i>infC-rpmI-rpmT</i>	Initiation factor 3	3.9
<i>ysbB</i>	<i>thrS-lytST-ysbAB</i>	Similar to unknown proteins	3.7
<i>kipI</i>	<i>ycsFGI-kipIAR-ycsK</i>	Inhibition of the autophosphorylation reaction of KinA	4.2
<i>kipR</i>	<i>ycsFGI-kipIAR-ycsK</i>	Transcriptional regulator (Ic1R family)	5.3
<i>ycsK</i>	<i>ycsFGI-kipIAR-ycsK</i>	Expression activated by TnrA and repressed by KipR; similar to unknown proteins	3.9

^a Function or comment in SubtiList database.

of the growth medium were made during growth of BD99, JC112, and JC112-R. During aerobic growth of bacteria on LB or semidefined media containing amino acids and nonfermentative organic acids, alkalinization of the medium occurs during the last half of the growth curve (46, 48, 54). The data in Fig. 3 demonstrate that growth of JC112 led to a smaller change in pH than growth of BD99. Restoration of the *tetL* gene in JC112-R resulted in only a marginal recovery of this property. Deletion of *rocR* increased the alkalinization observed with either the wild type or JC112-R (data not shown), consistent with indications from *E. coli* studies that different

FIG. 3. Alkalinization of the medium during growth. BD99, JC112, and JC112-R were grown in Spiz-KM medium (pH 8.0) for 6 h, to early stationary phase. Bars show the increase in pH determined in five independent experiments, with the error bars showing standard deviations. An analysis of variance, followed by pairwise comparison using the Bonferroni method, showed that the differences between BD99 and JC112 and between BD99 and JC112R were statistically significant ($P < 0.001$).

modes of amino acid catabolism are adaptive to different challenges of external pH (4, 5, 16, 38).

***citM* up-regulation and Co^{2+} sensitivity.** Previous studies failed to clarify the basis for the extreme Co^{2+} sensitivity of JC112 (51). The microarray experiments revealed up-regulation in JC112 of *citM*, which is capable of taking up citrate in complex with a number of divalent cations, including toxic divalent cations such as Co^{2+} (31, 33). We hypothesized that up-regulation of *citM* in JC112 could facilitate use of citrate to offset the reduced use of malate caused by the down-regulation of *maeN*, since the Spiz-KM medium used routinely in growth experiments contained both malate and citrate. This relationship was shown explicitly by growing BD99, JC112, and JC112-R in medium containing malate plus citrate, malate alone, or citrate alone (Fig. 4A). Growth of JC112 or JC112-R was still observed on malate alone, but the yield was much lower than on malate plus citrate, which was not the case with the wild-type strain. This presumably reflects the repression of *maeN*. Also, in contrast to the case for the wild type, the growth yields of JC112 and JC112-R on citrate alone were higher than those on malate alone, which correlated with increased expression and use of *citM*.

The overexpression of *citM* immediately provided an explanation for the extreme Co^{2+} sensitivity of JC112. The increased use of *citM* would, as a by-product, also result in increased import of Co^{2+} in a Co^{2+} -citrate symport reaction when Co^{2+} is added to the medium. Thus, we predicted that deletion of the *citM* gene in JC112 would restore Co^{2+} resistance. The *citM* genes of the wild-type and JC112 strains were

FIG. 4. Carbon source-dependent growth patterns and effects of deleting *citM* on growth yield and Co^{2+} sensitivity. A. BD99, JC112, and JC112-R were grown in Spiz-KM (pH 7.0) to which only the indicated carbohydrate carbon sources were added. Growth yield, measured by A_{600} , was recorded after 16 h. B. BD99 (black bars), BD99 Δ *citM* (open bars), JC112 (hatched bars), and JC112 Δ *citM* (stippled bars) were grown for 8 h on Spiz-KM with no additions or containing 200 μM added CoCl_2 . All experiments were conducted at least twice in duplicate. Error bars indicate standard deviations.

substituted with a Pm resistance cassette. The data in Fig. 4B show that the predicted link between *citM* and Co^{2+} sensitivity was correct. The growth yield of JC112 in the presence of 200 μM Co^{2+} was negligible, but it was substantially restored when the *citM* gene was deleted.

DISCUSSION

The new *tetL* mutants constructed in this study differed from the earlier *tetL* deletion strains represented by JC112 and JC112C in having a smaller deletion and being selected on LBK medium instead of Spiz-KM medium (7). Both sets of mutants exhibited the same phenotypes in a range of magnitudes. These phenotypes consistently included Co^{2+} sensitivity (Table 2), a phenotype that has no obvious relationship to the catalytic or physiological functions of TetL. The results support the conclusion that deletion of the *tetL* gene itself is responsible for the multiple phenotypes. Although in both sets of *tetL* deletion strains the small *tetL* leader sequence that is upstream of the *tetL* CDS is also deleted, that sequence is apparently involved solely in translational regulation of *tetL* (49). The current experiments further show that return of *tetL* to its original chromosomal position does not reverse the Na^+ and alkali sensitivity or the Co^{2+} sensitivity of the *tetL* deletion strains. The microarray analyses of JC112, JC112-R, and wild-type *B. subtilis* strongly support the explicit hypothesis that secondary mutations arise upon *tetL* deletion, ameliorating the physiological consequences of the mutation. Presumably, the significant genomic changes and consequent patterns of gene expression in the mutants account for the failure of *tetL* reintroduction into the chromosome to restore a wild-type phenotype. Yet, we did not experience difficulty in isolating *tetL* deletion mutants or discern a low frequency of their selection in side-by-side experiments in which the *amyE* gene was deleted (J. Jin and D. H. Bechhofer, unpublished data). The mechanism whereby mutations arise at high frequency under stress conditions is the focus of investigation in other laboratories (17, 25, 44) and is not addressed in our study.

The microarray analyses and the correlated physiological experiments also indicated specific ways in which changes in expression of ion-coupled membrane transporters would be

adaptive in JC112. The activities of these transporters are summarized diagrammatically in Fig. 5A. Loss of functional TetL simultaneously reduces Tc and Na^+ efflux capacity and both the H^+ and K^+ accumulation capacity, resulting in the alkali and Na^+ sensitivity. Without compensation, the cumulative results of these defects would probably be dire, since their individual effects would act synergistically in causing stress on the cells, in two ways. First, Na^+ cytotoxicity increases with increasing cytoplasmic pH, so a deficit in alkaline pH homeostasis makes otherwise subinhibitory Na^+ concentrations inhibitory, and elevated cytoplasmic Na^+ reduces the upper pH limit for growth (41, 42). Second, the cytotoxicity of Na^+ is more closely related to the Na^+/K^+ ratio than to the concentration of Na^+ itself (23). Although the basis for reduced expression of *nrgAB* in JC112 and JC112-R was not clarified, reduced NrgA function would be adaptive, assuming this protein functions analogously to its *E. coli* homologue, AmtB, as an NH_3 gas channel (30, 47, 57). The residual pH homeostasis of JC112 that is provided by other *B. subtilis* Na^+ (K^+)/ H^+ antiporters, such as NhaC and Mrp (27, 51; see below), would be adversely affected by NH_3 because it would consume cytoplasmic H^+ accumulated by these antiporters as it comes into equilibrium with its protonated NH_4^+ form in the relatively acidified cytoplasm (30, 43). Reduced malate uptake by MaeN would be adaptive because it lowers entry of Na^+ . The two alternate malate uptake systems of *B. subtilis* that account for growth of JC112 on malate are MleN and CimH. They achieve Na^+/H^+ antiport and net H^+ uptake, respectively, during malate uptake (32, 34, 53) (Fig. 5B). Use of MleN would ease both the Na^+ efflux and H^+ accumulation deficits, and use of CimH would ease the H^+ accumulation defect. Although the alternate malate transporters do not support yields of JC112 growth on malate alone that are comparable to wild-type levels (Fig. 4A), the increased activity of CitM and the arginine uptake proteins RocC and RocE provide additional carbon sources without coupling to Na^+ . Transport of amino acids by RocC and RocE is probably coupled to H^+ uptake, providing an added benefit. Increased acid production from metabolism of arginine, and perhaps other amino acids, could be an additional strategy of JC112 to compensate for its poor pH homeostasis capacity. Taken together,

FIG. 5. Ion-coupled membrane transporters that are hypothesized to change adaptively upon *tetL* deletion from *B. subtilis*. A. Schematic representation of the transport activities of JC112 that are deleted (*tetL*), down-regulated (*nrgA* and *maeN*), and up-regulated (*citM*, *rocC*, and *rocE*). Also shown is the change in *czcD* and *nhaC*, whose up-regulation was below the threshold of the current study but was shown earlier by transcriptional fusion and Northern blot analyses (51). B. Schematic representation of the transport activities of the two alternate L-malate transporters (34), MleN (53) and CimH (31, 32), that are hypothesized to take over for reduced *maeN* in support of the remaining capacity of JC112 for growth on malate. See text for discussion.

the increased CitM, RocC, and RocE function; decreased MaeN and NrgAB function; compensatory use of alternate malate uptake systems; and increased acid production from amino acid catabolism in JC112 could be critical adaptations to the major defect in pH homeostasis and the accompanying Na^+ sensitivity that result from loss of *tetL*. One of those changes, the elevated expression of CitM, was shown in this study to explain the Co^{2+} phenotype of *tetL* deletion mutants, a conclusion strongly supported by the greater Co^{2+} resistance of JC112 when *citM* was deleted (Fig. 4B).

Elevated expression of *czcD* and *nhaC* was found earlier in JC112 and JC112C, although these increases were below the threshold for this study (51). CzcD is an antiporter that effluxes divalent metal ions, including Co^{2+} , in exchange for H^+ and K^+ (22, 51), and NhaC is a Na^+/H^+ antiporter (51) (Fig. 5A). The increase in *czcD* expression was pronounced only when Co^{2+} was included in the medium (51), and the levels of CzcD in the membrane would be predicted to similarly increase when excess Co^{2+} is taken up by the high CitM activity of JC112. The $\text{Co}^{2+}/\text{H}^+\text{-K}^+$ antiport activity of CzcD would then concomitantly help reduce the levels of CitM-mediated Co^{2+} accumulation in the cytoplasm, while bringing H^+ and K^+ inward, mitigating the alkali sensitivity and diminished K^+ acquisition capacity of *tetL* deletion strains (Fig. 5A). Deletion of *czcD* was earlier found to exacerbate the K^+ acquisition

phenotypes of *tetL* deletion strains JC112 and JC112C (51). Increased Na^+/H^+ activity of the NhaC and Mrp antiporters probably facilitates residual cytoplasmic pH homeostasis in the absence of TetL (8, 27, 42).

Finally, this study did not identify master genes whose altered activity accounts for the pattern of altered gene expression observed in JC112 and JC112-R. The genes that are up- or down-regulated might constitute a regulon that is coordinately controlled by a combination of stresses, e.g., elevated cytoplasmic pH and/or Na^+ (or the Na^+/K^+ ratio). However, there is no evidence to date supporting such coordinated regulation. Current information about the genes and operons whose expression is altered in JC112 and JC112-R indicates that they are regulated by a variety of global and specific regulators. For example, some of the affected genes in JC112 are a subset of the members of the SigL regulon (*rocA*, *rocD*, *rocG*, and *acoA*) (24), some are regulated by CcpA (e.g., *rocG*, the *rbs* operon, *treA*, *treP*, and *gltAB*) (2, 40, 50), and regulation of both *citM* and *maeN* involves individual two-component systems (34). Analysis of global gene expression in *tetL* deletion strains other than JC112 would show whether the cellular response to the lack of Tet(L) is a conserved one. This finding would suggest the existence of one or a limited number of controlling genes that can act to coordinate the cellular reaction to the loss of a major Na^+ and pH regulator.

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