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Response of *Sinorhizobium meliloti* to Elevated Concentrations of Cadmium and Zinc▽†

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Whole-genome transcriptional profiling was used to identify genes in *Sinorhizobium meliloti* 1021 that are differentially expressed during exposure to elevated concentrations of cadmium and zinc. Mutant strains with insertions in metal-regulated genes and in genes encoding putative metal efflux pumps were analyzed for their metal sensitivities, revealing a crucial role for the SMc04128-encoded P-type ATPase in the defense of *S. meliloti* against cadmium and zinc stress.

Transcriptional profiling was used to characterize genes that *Sinorhizobium meliloti* differentially expresses to cope with elevated concentrations of heavy metals. The response to a toxic metal, cadmium, was compared with the response to an essential metal, zinc. In general, cells face a dilemma: on one hand, they need efficient uptake systems for essential metal ions that often exist only in trace concentrations in the environment, but on the other hand, nonspecific uptake systems might allow the influx of toxic metal ions. To cope with metal ion excess, cells employ efficient efflux systems, and a multitude of genes encoding efflux proteins have been identified in the genomic sequences of *Bacteria* and *Archaea* (14). To analyze the role of metal ion efflux and the roles of genes that were differentially expressed in the presence of elevated concentrations of cadmium and zinc in microarray experiments, mutant strains with insertions in these genes were analyzed for their phenotypes regarding metal sensitivity.

Transcriptional whole-genome profiling of *S. meliloti* in response to cadmium and zinc. Transcriptional profiling with whole-genome microarrays was used to analyze the global response of *S. meliloti* to metal stress. *S. meliloti* cells growing in GTS minimal medium (11) to early logarithmic phase were exposed for 2 hours to 50 μM $CdCl_2$ or 100 μM $ZnSO_4$. In order to stress, but not kill, the cells, the concentrations chosen represented 50% of the MICs of cadmium and zinc (Cd, 100 μM; Zn, 200 μM) in GTS medium for *S. meliloti* (data not shown). The 2-hour exposure time was selected in order to be able to monitor the early to intermediate responses of *S. meliloti* to metal stress without having to account for a lower growth rate in the metal-treated cultures. After 2 hours of incubation, a slight increase in growth was observed in all cultures: the average increases in the optical density at 600 nm were 0.03 in the cadmium-treated culture, 0.04 in the zinc-treated culture, and 0.06 in the control culture ($N = 3$; standard error of the mean, <0.007).

Using a cutoff value for M [$M = \log_2$ (experimental signal/control signal)] of ≥1.58 or ≤−1.58 (a threefold difference) and a P value of ≤0.05, a total of 72 genes were found to be differentially expressed when cells were exposed to Cd(II). Of these genes, 66 were up-regulated and 6 were down-regulated (see Table S1 in the supplemental material). Exposure to Zn(II) resulted in the identification of 53 differentially expressed genes: 39 were up-regulated and 14 down-regulated (see Table S2 in the supplemental material). Of the genes up-regulated by Cd(II), 59% were located on either the pSymA or pSymB plasmid, and 90% of the genes up-regulated by Zn(II) were either pSymA or pSymB encoded. This pattern shows a strong bias for a plasmid location of the up-regulated genes, especially for zinc. In comparison, the distribution of all protein-coding genes in *S. meliloti* is 21, 25, and 54% for pSymA, pSymB, and the chromosome, respectively (8). A similarly strong bias for a pSymB location was reported for *S. meliloti* genes up-regulated during osmostress (6) and genes controlled by the stress-responsive RpoE2 sigma factor (18).

Genes regulated by cadmium. Genes up- or down-regulated by cadmium are shown in Table S1 in the supplemental material. Among the genes most highly expressed under cadmium stress were those encoding efflux pumps, in particular, SMc01095 (*mexF1*) and SMb20345, both of which encode cation/proton antiporter proteins of the RND family and which showed 41- and 4-fold induction, respectively. Predicted to form an operon with *mexE1F1* (http://www.microbesonline.org/) and located directly upstream of it is the SMc01093 gene, which showed 20-fold induction. Some well-described stress response genes are induced by Cd(II), including the gene encoding a small heat shock protein of the Hsp20 family (SMb21294), the *katA* catalase gene, and the adjacent regulatory *oxyR* gene. These genes exhibited 6-, 22-, and 6-fold induction, respectively. Also induced by Cd(II) is SMc02576, located between and in the same frame as *hslV* and *hslU*, both of which encode protease subunits functioning in the heat shock response in *Escherichia coli* (20). A specific oxidative-stress response gene induced by Cd(II) is SMa1894, which

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encodes a protein with similarity to MsrB (methionine sulfoxide reductase), involved in reversing methionine oxidation (7). Two other genes involved in biosynthesis of sulfur-containing amino acids were up-regulated during cadmium stress, the *metH* gene encoding methionine synthase, which carries out the final step in methionine synthesis, and SMc02882, encoding an uncharacterized protein involved in cysteine biosynthesis. Nine cadmium-induced genes appear to encode proteins involved in oxidoreduction reactions (see Table S1 in the supplemental material), and others have putative functions in electron transport, such as the *cycF* and *cycG* genes. The *cycFG* genes are predicted to form an operon and were nine- and threefold induced by Cd(II), respectively. The deduced proteins of both genes display N-terminal signal sequences and cytochrome *c*-type heme binding sites; CycF has one and CycG has two. The *ccsA* gene, involved in cytochrome *c* biogenesis (22), was fourfold up-regulated by Cd(II). Also worth mentioning is the 20-fold induction of the *cah* gene, encoding a probable carbonic anhydrase with a cleavable N-terminal signal sequence. Carbonic anhydrases are enzymes that interconvert CO_2 and bicarbonate and generally use zinc as a cofactor, but recently, carbonic anhydrases of marine diatoms have been described that contain cadmium in their metal centers (12). It is worth mentioning that several genes encoding proteins predicted to contain zinc as a cofactor (carbonic anhydrase, *metH* [10], SMa0308, and SMa2383) were up-regulated by cadmium. Finally, there are three adjacent genes that were highly induced by Cd(II), *nex18*, SMa1078, and *tspO*, with 16-, 9-, and 6-fold induction, respectively. The *nex18* gene has been found to be highly expressed in developing bacteroids, and its gene product shows a fasciclin domain that is typical of a class of surface-associated proteins involved in cell adhesion (15). The *nex18* mutants are impaired in symbiosis (15). The *tspO* gene was identified during a screen for genes that regulate *ndi*, a locus induced by nutrient deprivation in *S. meliloti* (3). The *S. meliloti tspO* gene product is 42% identical and 67% similar to the tryptophan-rich sensory protein TspO of *Rhodobacter sphaeroides*. The *R. sphaeroides* TspO protein has been shown to be an outer membrane protein and a negative regulator of photosynthesis genes in response to light and oxygen, probably by binding to an intermediate in tetrapyrrole synthesis (19). Among the six genes found to be down-regulated by Cd(II) were the *serC* gene, encoding a protein involved in serine biosynthesis; the *mcpU* gene, encoding a probable chemoreceptor involved in chemotaxis; a gene encoding a putative ABC transporter subunit (SMc02337); and a gene encoding a TonB-like periplasmic protein (SMc01515).

Genes regulated by zinc. Genes up- or down-regulated by zinc are shown in Table S2 in the supplemental material. A large proportion of genes induced by zinc encode putative transporters, mainly of the ABC type (SMa0270, SMb21219, and SMb21344), and those putatively involved in the transport of C_4 dicarboxylates (SMa0157), amino acids (SMb21507), or divalent heavy-metal ions (SMb20011). In addition, several genes encoding proteins involved in electron transport were induced by zinc stress, including the above-mentioned *c*-type cytochrome genes *cycFG*, as well as the *cyoA* and *cyoC* genes encoding putative cytochrome *o* ubiquinol oxidase subunits. Located in the vicinity of *cyoABC* and also up-regulated are SMb21484, encoding the putative extracytoplasmic sigma factor RpoE5, and SMb21490, encoding a putative SUR1-like protein, which is similar to the *shb1* gene product involved in cytochrome aa_3 biogenesis in *Bradyrhizobium*. The highest up-regulation during zinc exposure (14-fold) was exhibited by the SMb21211 gene, which encodes a putative membrane-associated, metal-dependent hydrolase. Also highly induced by Zn(II) were the above-mentioned *nex18*, SMa1078, and *tspO* genes (nine-, three-, and fivefold induced, respectively). Interestingly, a gene involved in exopolysaccharide synthesis, the *exoK* gene, was induced fivefold by zinc.

Among the genes down-regulated by Zn(II) was the *cysK1* gene, encoding cysteine synthase, which carries out the last step in cysteine biosynthesis. CysK1 shares 69% identical and 82% similar amino acids with the deduced protein product of *cysK* of *Azospirillum brasilense*, on which it confers tellurite resistance (17). Also down-regulated were the *uvrB* gene, encoding the central component of the nucleotide excision repair system UvrABC, and SMc02797. The deduced gene product of SMc02797 displays amino acids 40% identical and 53% similar to those of the *E. coli thdF* (*trmE*) gene product. The *E. coli trmE* gene encodes a molecular-switch GTPase, which is involved in tRNA modification, regulation of ribosome function (2), and regulation of glutamate-dependent acid resistance (9). The *S. meliloti thdF* (*trmE*) gene is located in a region important for regulation of transcription and cell division, since the region contains the gene encoding the transcription termination factor Rho, the glucose-inhibited division genes *gidAB*, and the chromosomal partitioning genes *parAB*.

Validation of the results from the microarray experiments by qRT-PCR. To verify the results from the microarray experiments, we selected 10 genes that had shown induction or repression in the presence of both metal ions, cadmium and zinc. Oligonucleotides used as primers in the quantitative real-time PCR (qRT-PCR) experiment are shown in Table S3 in the supplemental material. The qRT-PCR results agreed with the microarray results in all cases (Pearson's correlation coefficient, $r = 0.93$), although the induction values for the up-regulated genes were measured at a higher level with qRT-PCR than with the microarrays (see Table S4 in the supplemental material; Fig. 1).

Analysis of mutant strains. We tested the hypothesis that genes that respond with differential expression to the presence of elevated levels of cadmium and zinc are involved in defending organisms against these heavy-metal ions. We obtained *S. meliloti* mutant strains containing mini-Tn*5* transposons (16) in some of the genes that showed more than threefold up- or down-regulation in the microarray experiments (see Tables S1 and S2 in the supplemental material). As a preliminary screen, the mutant strains were first tested for cadmium or zinc sensitivity using a disk diffusion assay (13). Mutants that showed increased or decreased sensitivity in the disk diffusion assay were further tested for the ability to grow in liquid culture with increasing metal concentrations (MIC determination). Whereas the disk diffusion assay revealed only minor variations of cadmium and zinc sensitivity for the 47 mutant strains tested in comparison to the wild type, the *exoK* mutant showed consistently increased sensitivity to zinc, but not to cadmium, in the disk diffusion and MIC assays (Fig. 2A and B). The ExoK endo-1,3-1,4-β-glycanase is involved in generating low-molecular-weight succinoglycans by cleaving nascent succino-

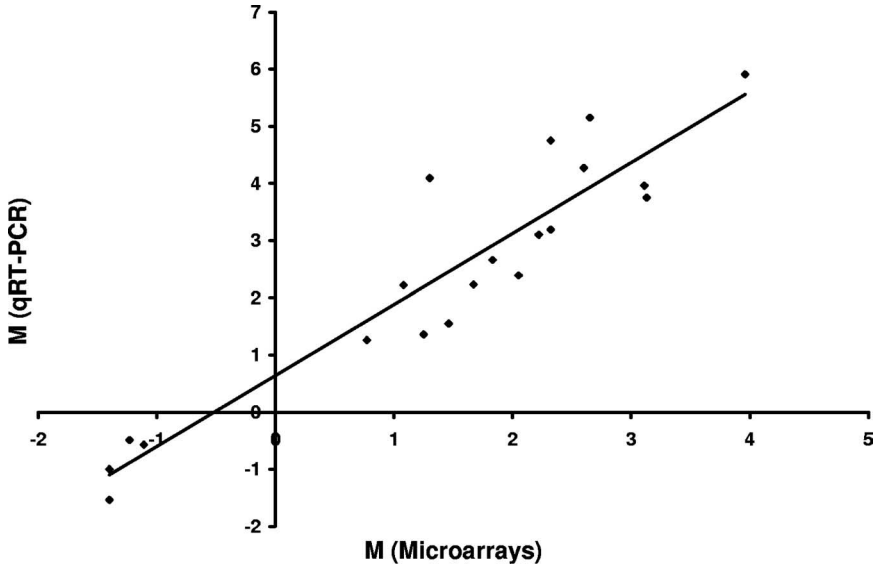

FIG. 1. Correlation of microarray and qRT-PCR results. *M* values calculated from the microarray and the qRT-PCR experiments were plotted; $r^2 = 0.87$.

glycan chains (21). One explanation for the increased zinc sensitivity of the *exoK* mutant could be that in the wild type, the zinc cations bind to the negatively charged succinoglycans on the outside of the cell, and therefore, the zinc ions are sequestered and do not reach the cytoplasm. The succinoglycans could also act as a general diffusion barrier, since *exo* mutants lacking succinoglycans completely (*exoY* mutants) have been found to exhibit increased H_2O_2 sensitivity (4).

Another strain that consistently displayed increased sensitivity to cadmium and zinc in the disk assay, as well as in the MIC assay, is the *ccsA* mutant (Fig. 2A and B). Yurgel et al. (22) noted a 50% identity of CcsA with the *Rhodobacter capsulatus* CcdA protein. CcdA is required for cytochrome *c* biogenesis and has been postulated to relay cytoplasmic reducing equivalents to apocytochromes in the periplasm (5). This confirms the view that cadmium binds to or interferes with the thiol groups of proteins. During exposure to elevated concentrations of cadmium, when Cd(II) binds to thiol groups of proteins, more reducing equivalents would be needed in the periplasm, so the cell would respond with an up-regulation of the *ccsA* gene to produce more CcsA protein. Based on the important role of *c*-type cytochromes in electron transport, it is not surprising that a pleiotropic phenotype has been described for the *S. meliloti ccsA* mutant, including slow growth in rich, but not in minimal, medium (22). Although we occasionally observed a lower growth rate for the *ccsA* mutant, the results in Fig. 2 show that this mutant strain reached optical densities similar to those of the wild-type strain after 48 h, when no metal or low concentrations of metal were added to the rich tryptone-yeast extract (TY) medium.

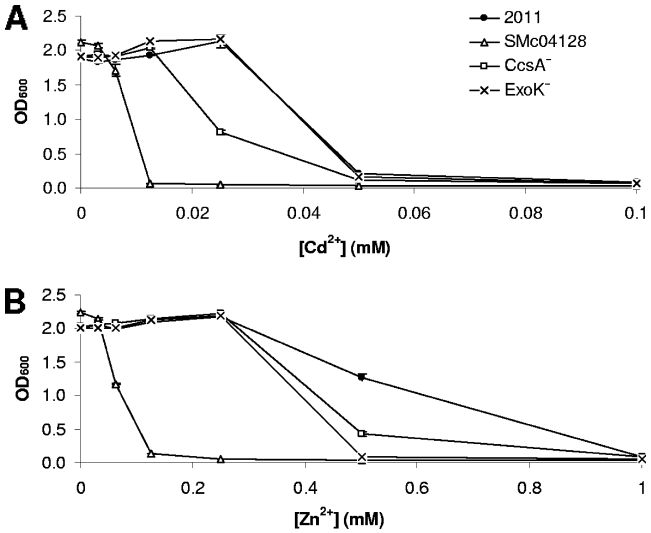

FIG. 2. Effects of increasing metal ion concentrations on growth of *S. meliloti* wild-type strain 2011 and the *ccsA*, *exoK*, and SMc04128 mutant strains in TY liquid medium. The absorbance (optical density at 600 nm [OD_{600}]) was determined after 48 h of growth in the presence of cadmium (A) or zinc (B). The data points represent the means of triplicate cultures from one typical experiment, and the error bars denote the standard errors of the mean.

Thus, only a couple of genes that were found to be significantly up- or down-regulated in the microarray experiments seem to play roles in protecting the bacterial cell against the toxicity of elevated concentrations of cadmium and zinc during growth. For many bacterial species, it has been shown that efficient efflux pumps play a major role in heavy-metal defense (14). Table S5 in the supplemental material shows a compilation of putative metal efflux proteins that were found to display similarities to characterized metal efflux proteins of *Ralstonia metallidurans* and *Staphylococcus aureus*. Mutant strains with transposon insertions in the putative efflux genes were obtained (16). Of 18 mutant strains tested, 2 showed extreme cadmium and zinc sensitivity. In both of these strains, the transposon insertion was located in SMc04128, which encodes a heavy-metal transport P_{1B}-type ATPase of the IB-2 subgroup (1). These two mutant strains displayed by far the most ex-

treme phenotypes in the disk diffusion assay, and the MICs of cadmium (0.0125 mM) and zinc (0.125 mM) in TY medium were substantially lower for the mutants than for the wild-type strain (Cd, 0.1 mM; Zn, 1 mM) (Fig. 2A and B). In addition, for both SMc04128 mutants strains, slightly increased sensitivities to copper(II), lead(II), nickel(II), and cobalt(II) were observed (data not shown). These data show that the SMc04128-encoded P-type ATPase plays a crucial role in the defense of *S. meliloti* during growth in high concentrations of cadmium and zinc, probably by catalyzing efficient efflux of Cd(II) and Zn(II) ions.

In conclusion, transcriptomic studies with *S. meliloti* exposed to elevated concentrations of cadmium revealed that the cell reacts with the induction of genes that encode efflux pumps of the RND family (*mexF1* and SMb20345) and genes that are involved in oxidative (*katA*, *oxyR*, and *msrB*) or general (*hsp20* and SMc02576) stress response. Less stress response was observed when *S. meliloti* was exposed to elevated concentrations of zinc, but genes that encode putative ABC-type transporters (SMa0270, SMb21219, and SMb21344) were induced. The analysis of mutant strains revealed that ExoK and CcsA play minor roles and the SMc04128-encoded P-type ATPase plays a major role in the defense of *S. meliloti* against high concentrations of cadmium and zinc.

Microarray data accession number. Detailed protocols for the microarray experiments, as well as the raw data, have been deposited in the ArrayExpress database with the accession number E-MTAB-13 (http://www.ebi.ac.uk/arrayexpress/).

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