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ORIGINAL PAPER

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# New genes involved in chromate resistance in *Ralstonia metallidurans* strain CH34

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**Abstract** Chromate resistance in *Ralstonia metallidurans* CH34 is based on chromate efflux catalyzed by ChrA efflux pumps. The bacterium harbors two chromate resistance determinants, the previously known $chr_1$ on plasmid pMOL28 (genes *chrI*, $chrB_1$, $chrA_1$, *chrC*, *chrE*, $chrF_1$) and $chr_2$ on the chromosome (genes $chrB_2$, $chrA_2$, $chrF_2$). Deletion of the genes *chrI*, *chrC*, $chrA_2$, $chrB_2$ and $chrF_2$ influenced chromate resistance and transcription from a $chrBp_1$::*lacZ* fusion. Deletion of the plasmid-encoded gene $chrB_1$ did not change chromate resistance or $chrBp_1$ regulation. Northern hybridization and primer-extension experiments were used to study transcription of the plasmid-encoded $chr_1$ determinant. Transcription of $chrB_1$, $chrA_1$ and *chrC* was induced by chromate. The presence of sulfate influenced transcription positively. The $chrBp_1$, $chrAp_1$ and *chrCp* promoters showed some similarity to heat-shock promoters. Transcription of the gene *rpoH* encoding a putative heat-shock sigma factor was also induced by chromate, but *rpoH* was not essential for chromate resistance. The ChrC protein was purified as a homotetramer and exerted superoxide dismutase activity. Thus, possible regulators for chromate resistance (ChrI, $ChrB_1$, $ChrB_2$, $ChrF_1$, and $ChrF_2$) and an additional detoxification system (ChrC) were newly identified as parts of chromate resistance in *R. metallidurans*.
**Electronic supplementary material** to this paper can be obtained by using the Springer LINK server located at http://dx.doi.org/10.1007/s00203-002-0492-5.

**Keywords** Chromate resistance · *Ralstonia metallidurans* · *Alcaligenes eutrophus*

**Abbreviations** *SOD* Superoxide dismutase

## Introduction

*Ralstonia metallidurans* strain CH34 [formerly *Alcaligenes eutrophus* CH34, (Goris et al. 2001)] contains at least eight determinants encoding resistance to toxic heavy metals, located either on the bacterial chromosome or on one of the two indigenous megaplasmids pMOL28 and pMOL30 (Nies 1999, 2000). *R. metallidurans* strains AE128(pMOL30) and AE126(pMOL28) contain only one of the two megaplasmids, respectively, and strain AE104 is plasmid-free (Mergeay et al. 1985). On plasmid pMOL28, two inducible metal resistance determinants are located adjacent to each other: the *cnr* determinant encodes resistance to $Co^{2+}$ and $Ni^{2+}$ and is physiologically based on metal-cation efflux (Liesegang et al. 1993). The *chr* determinant mediates resistance to chromate (Nies et al. 1989a, 1990), the mechanism of which is based on a decreased accumulation of chromate (Nies and Silver 1989b), probably by $CrO_4^{2-}$ efflux (Alvarez et al. 1999; Pimentel et al. 2002).

There are three open reading frames (ORFs) in the original sequence of the 2.6-kb *Eco*RI *chr* fragment from plasmid pMOL28 (Fig. 1A): *chrB*, *chrA* and a truncated *ORF3* (now the 5′ end of *chrC*), which seemed not to be essential for chromate resistance (Nies et al. 1990). Since a second *chr* determinant was identified in the *R. metallidurans* genome [preliminary sequence data were obtained from the DOE Joint-Genome Institute (JGI) at http://www.jgi.doe.gov/JGI_microbial/html/ralstonia/ralston_homepage.html], the already described pMOL28 genes were renamed $chrB_1$, $chrA_1$ ($chr_1$ determinant) and the newly found homologs $chrB_2$ and $chrA_2$ ($chr_2$ determinant).

$ChrA_1$ is a membrane-bound protein (Nies et al. 1998) and probably responsible for chromate efflux (Alvarez et al. 1999; Pimentel et al. 2002). The chromate resistance

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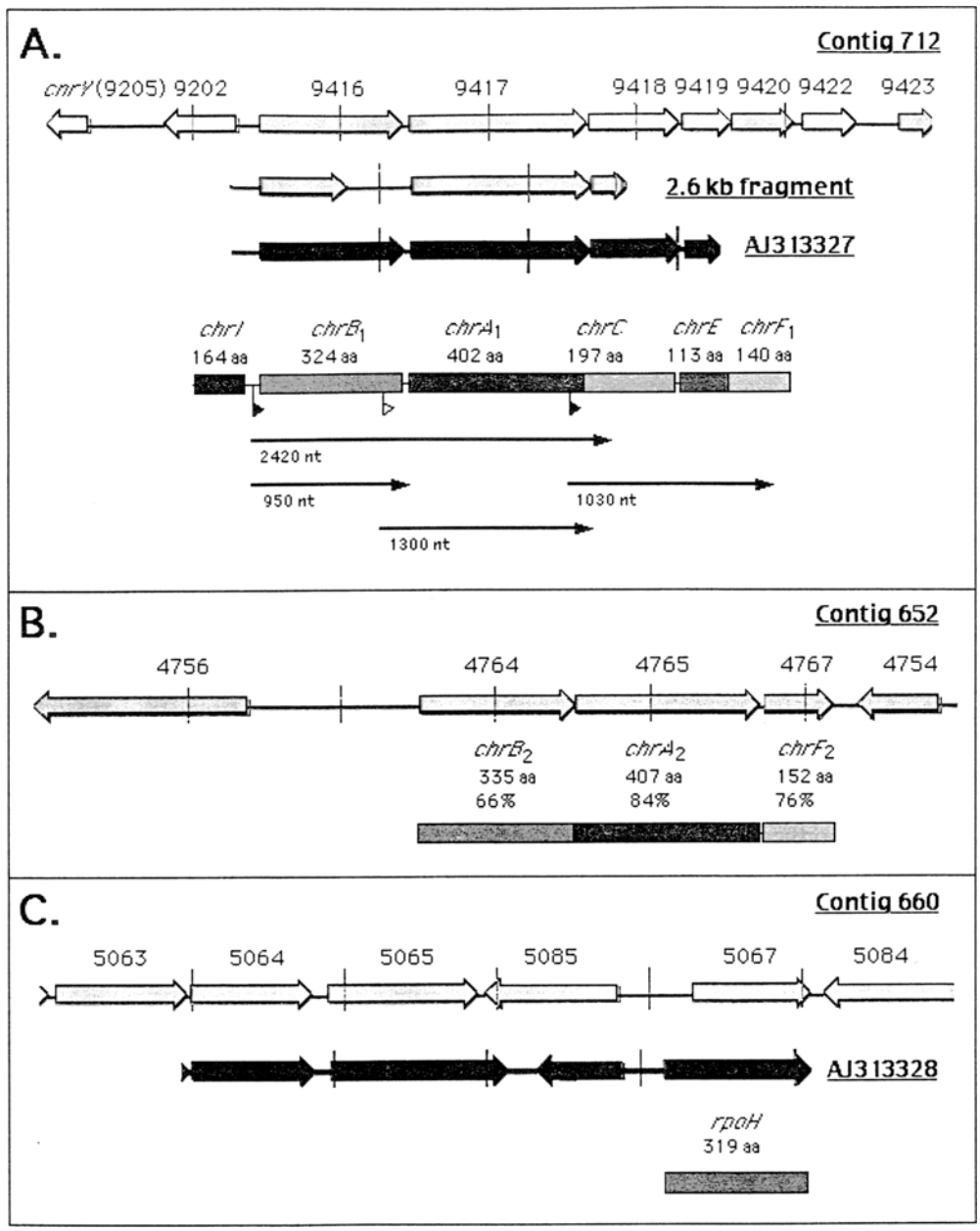


**Fig. 1A–C** Genes involved in chromate resistance in *Ralstonia metallidurans* CH34. **A–C** Regions on megaplasmid pMOL28 (**A**) or the bacterial chromosome (**B**, **C**) involved in chromate resistance. The chains of *boxed arrows* indicate the position of ORFs as determined by the *R. metallidurans* genome project at DOE Joint-Genome Institute. The gene numbers and the respective contig are indicated above the *boxed arrows*. **A** The ORFs in the published 2.6-kb *Eco*RI fragment are shown (Nies et al. 1990). **A**, **C** *Black boxed arrows* indicate the *chr* genes and their direction of transcription in the sequence of accessions AJ313327 and AJ313328. *Vertical bars* in all ORF maps are distance markers of 1 kb. The genes that are the subject of this publication are shown below the ORF map(s) as *gray bars* with the proposed gene names and the sizes of the predicted proteins (*aa* amino acid residue). In **A**, the position of $chr_1$ promoters (*filled arrowheads; open arrowhead*, a constitutive promoter) and the mRNAs determined by Northern analysis are also shown

region of *Pseudomonas aeruginosa* (Cervantes et al. 1990) harbors an ORF downstream of the respective *chrA* gene. The predicted product displays a high (45%) amino acid identity to the predicted *ORF3* product of *R. metallidurans* while the two ChrA proteins are only 29% identical (Cervantes et al. 1990). Since the *P. aeruginosa chr* determinant does not contain a *chrB* gene and is expressed constitutively, involvement of $ChrB_1$ in regulation of $chr_1$ has been suggested (Nies et al. 1990). In this report, nine genes that might be involved in chromate resistance in *R. metallidurans* are described and functions to their gene products are assigned.

## Materials and methods

### Growth conditions

Tris-buffered mineral salts medium (Mergeay et al. 1985), containing either 3 mM sulfate as published or 30 µM sulfate, was used to grow *R. metallidurans* strains AE126(pMOL28), the plasmid-free strain AE104, and derivatives of both strains. The strains were aerobically cultivated at 30 °C with shaking and harvested at the late-exponential growth phase. Analytical grade $K_2CrO_4$ was used to prepare a 1 M stock solution. Solid Tris-buffered media contained 20 g agar/l. Nutrient broth (NB, Difco) was used as complex medium for *R. metallidurans* and Luria broth for *Escherichia coli* (Sambrook et al. 1989). β-Galactosidase activity was determined in permeabilized cells as published previously (Nies 1992). One unit was defined as the activity forming 1 nmol of *o*-nitrophenol per min (Pardee et al. 1959; Ullmann 1984) at 30 °C.

### Genetic techniques

Standard molecular genetic (Sambrook et al. 1989) and previously published (Nies et al. 1987; Große et al. 1999) techniques were used. For conjugal gene transfer, overnight cultures of donor strain *E. coli* S17/1 (Simon et al. 1983) and of the *R. metallidurans* recipient strain AE104 grown at 30 °C in complex medium were mixed (1:1) and plated onto nutrient broth agar. After overnight growth, the bacteria were suspended in saline and plated onto selective media as previously described (Nies et al. 1987). DNA was sequenced using an automated A.L.F. DNA Sequencer (Pharmacia, Uppsala, Sweden) as described previously (Große et al. 1999) and analyzed with the BioTechnix 3d program (http://www.Biotechnix3D.com).

### Genomic sequence data

Preliminary sequence data were obtained from the DOE Joint–Genome Institute (DOE/JGI) at: http://www.jgi.doe.gov/JGI_microbial/html/ralstonia/ralston_homepage.html.

### Deletions

Deletions were designed such that polar effects were prevented, and carried out as published (Große et al. 1999). To mutate the pMOL28-encoded genes $chrB_1$, *chrC* and *chrI*, 300 base pairs (bp) upstream of the respective target gene were amplified by PCR from total DNA of *R. metallidurans* AE126(pMOL28) and cloned as *Xba*I-*Mun*I fragments. In addition, 300 bp downstream of the target gene were also amplified and cloned as *Xba*I-*Mun*I fragments, and the upstream fragments started with the last 27 bp of the respective gene. Both fragments were digested with *Mun*I, but not with *Xba*I, ligated into pGEM T-easy-vector (Promega, Madison, Wis., USA), and verified by DNA sequence analysis. The fragments from plasmid pGEM T-easy were cloned as *Xba*I fragments into pLO2 (Lenz et al. 1994) and used to delete the respective gene from plasmid pMOL28 by double cross recombination. Clones containing the correct mutation were screened by PCR followed by sequence analysis of the resulting fragment. Additionally, the mutations were verified by Southern DNA/DNA hybridization to exclude artifacts. The primer pairs used are listed in the electronic supplementary material (Table W1).

### Insertions

Mutation of the chromosomal genes $chrB_2$, $chrA_2$, $chrF_2$ and *rpoH* were achieved by insertions. A 300-bp fragment located in the middle of the target gene was amplified by PCR from total DNA of *R. metallidurans* AE104 and cloned into pLO2 (Lenz et al. 1994). The resulting plasmid was conjugated into *R. metallidurans* strains. Kanamycin resistance encoded by pLO2 was used to screen for insertion of pLO2 into the target gene by single cross recombination. Correct disruptions were verified by PCR.

**Table 1** Minimal inhibitory concentrations. Cells of *Ralstonia metallidurans* strain AE126(pMOL28) and its plasmid-free derivate strain AE104 were cultivated on solidified Tris-buffered mineral salts medium containing either 3 mM sodium sulfate or 30 µM sulfate and various concentrations of potassium chromate. Growth was analyzed after 3 days at 30 °C. Each determination was done at least three times with identical results

| Bacterial strain | Relevant genotype | MIC values (µM of chromate) | |
|---|---|---|---|
| | | 3 mM Sulfate | 30 µM Sulfate |
| AE126 | pMOL28 | 350 | 40 |
| | $\Delta chrB_1$ | 350 | 40 |
| | Δ*rpoH* | 350 | 40 |
| | Δ*chrC* | 300 | 35 |
| | Δ*chrI* | 300 | 35 |
| AE104 | Plasmid-free | 150 | 20 |
| | $\Delta chrF_2$ | 175 | 20 |
| | $\Delta chrB_2$ | 70 | 10 |
| | $\Delta chrA_2$ | 70 | 10 |

### Construction of the reporter gene fusions

Using the primers listed in the electronic supplementary material (Table W1), about 300 bp upstream of the ATG of $chrB_1$ and $chrA_1$ were amplified with PCR from plasmid pMOL28 DNA isolated from strain AE126(pMOL28). The DNA fragments were cloned upstream of a promoterless *lacZ* gene on the broad-host-range plasmid pVDZ'2 (Deretic et al. 1987). The resulting plasmids, pDNA355 (*chrA1p::lacZ*) and pDNA356 (*chrB1p::lacZ*), were conjugated into *R. metallidurans*.

### RNA techniques

For RNA isolation, cells of *R. metallidurans* strains AE126 (pMOL28) and AE104 were cultivated with shaking at 30 °C in Tris/gluconate medium (Mergeay et al. 1985) to the late-exponential phase of growth. The chromate concentration in the growth medium was 0 (uninduced) or 50 µM (to induce *chr*), and the sulfate concentration 3 mM (high sulfate) or 30 µM (low sulfate). Total RNA of *R. metallidurans* was isolated and Northern (RNA) blot analysis was carried out as published (Große et al. 1999). The probes for the Northern analysis were amplified with gene-specific DNA primers (Table W1, electronic supplementary material). For reverse transcription, the cells were cultivated in the presence of 50 µM chromate and 3 mM sulfate. Total RNA was isolated from those cells and digested with 1 U of RNAse-free DNAse I (Roche Diagnostics, Mannheim, Germany) per µg RNA for 2 h at 37 °C. Primer-extension analysis was done by modification of a standard protocol (Sambrook et al. 1989) using fluorescein-labeled oligonucleotides (Table W1,electronic supplementary material) and an automated A.L.F. DNA Sequencer (Pharmacia, Uppsala, Sweden) as described previously (Große et al. 1999).

### Purification of ChrC

The *chrC* gene was PCR-amplified from megaplasmid pMOL28 (Mergeay et al. 1985) DNA using the primers listed (Table W1, electronic supplementary material). The fragment was cloned into the vector plasmid pASK7 (IBA, Göttingen, Germany) providing the ATG start codon, the Strep-TagII to the amino-terminus and the *tetAp* promoter. Correct cloning was verified by DNA sequence analysis.

ChrC was expressed in *E. coli* strain BL21 (Stratagene Europe, Amsterdam, The Netherlands) by freshly transforming the bacteria with the pASK expression vector. The transformants were cultivated overnight at 30 °C, diluted 1:100 into 200 ml of fresh LB, and cultivated with shaking at 30 °C until the optical density at 600 nm reached 0.6. Expression of ChrC was induced with 200 µg anhydrotetracyclin/l and incubation was continued for 3 h. The cells were harvested by centrifugation (8,000 rpm, 30 min, 4 °C), the pellet was suspended in 30 ml buffer W (100 mM Tris-HCl, pH 8.0) and disrupted twice with a French press (SLM Aminco, SOPRA, Germany, 8.6 MPa) in the presence of the protease inhibitor phenylmethylsulfonyl fluoride (1 mM) and DNase (10 µg/ml). Debris was removed by an additional centrifugation. The soluble fraction was isolated by ultracentrifugation (100,000×*g*, 3 h, 4 °C) and applied to a streptactine agarose column (bed volume 3 ml), which was washed with 30 ml of buffer W. ChrC was finally eluted with 100 mM Tris-HCl buffer (pH 8.0) containing 2.5 mM desthiobiotin.

Superoxide dismutase activity stain

Superoxide dismutase (SOD) activity was determined by the method of Beauchamp and Fridovich (1971). Five µg of purified ChrC protein was applied onto a native 8% polyacrylamide gel. Following electrophoretic separation, the gel was incubated in the dark with 25 ml of a solution containing per liter of water: 250 mg nitro blue tetrazolium chloride (N6876, Sigma, Taufkirchen, Germany) and 100 mg riboflavin (R4500, Sigma). Staining was developed in the daylight after the addition of 25 µl of TEMED (T8133, Sigma). For specific inhibition of Fe-SOD activity, the gel was stained in the presence of 0.5 g $H_2O_2$/l as published (Roux and Coves 2002).

PhoA fusions

The 5′ segment of the $chrB_1$ gene was amplified by PCR (primer listed in Table W1, electronic supplementary material) and cloned into a derivative of the *phoA* fusion vector pECD500 (Rensing et al. 1997) in *E. coli* CC118 (Manoil 1990). Specific activity of alkaline phosphatase (Manoil 1990) was determined in triplicate as described previously (Nies et al. 1998). Control values were *phoA* fusions of a leader-free β-lactamase gene (negative) and a β-lactamase gene encoding the full leader sequence (positive control) (Rensing et al. 1997).

## Results

### Genes putatively involved in chromate resistance in *R. metallidurans*

One of the two megaplasmids of *R. metallidurans*, pMOL28, harbors resistance to chromate (Nies et al. 1989a). The plasmid pMOL28-encoded chromate resistance determinant $chr_1$ was initially cloned together with the cobalt-nickel resistance determinant *cnr* on cosmid pDNA206 (Nies et al. 1989a) as part of a pMOL28 gene bank (Nies et al. 1989a). The $chr_1$ determinant was further subcloned on a 2.6-kb fragment (Nies et al. 1990) that contained the genes $chrB_1$, $chrA_1$ and *ORF3* (now the 5′ end of *chrC*, Fig. 1A). The nucleotide sequence downstream of $chrA_1$ was determined and compared with the genomic sequence of *R. metallidurans* [preliminary sequence data were obtained from the DOE Joint–Genome Institute (DOE/JGI)]. Regions containing difference in the sequences were resequenced and the information used to update a previous submission of the $chr_1$ region [(Nies et al. 1990), EMBL submission AJ313327, Fig. 1A].

In the updated version, the size of the predicted $ChrB_1$ protein increased to 324 amino acids (Fig. 1A). The *chrC* gene directly downstream of $chrA_1$ encoded a putative product of 197 amino acid that was homologous to manganese- or iron-SODs. The highest identity, 34%, was observed with a putative SOD (annotation number TVN0061) from *Thermoplasma volcanium*. Directly downstream of *chrC* were two more ORFs encoding putative proteins of 113 amino acids and 140 amino acids, respectively. These genes were designated *chrE* and $chrF_1$ (Fig. 1A). The next ORF downstream of $chrF_1$ (gene 9422, Fig. 1A) was more than 400 bp apart from the 3′ end of $chrF_1$ and was therefore not considered for further analysis. Upstream of $chrB_1$, but in the opposite orientation of transcription, was another ORF (*chrI*) that encodes a putative protein of 164 amino acids. This gene was followed by the *cnrY* regulatory gene of the cobalt-nickel resistance determinant *cnr* (Fig. 1A). Thus, in addition to the known genes $chrB_1$ and $chrA_1$, the $chr_1$ determinant on plasmid pMOL28 may contain at least four more genes that might be involved in chromate resistance.

The DNA sequence (EMBL submission AJ313328) of a fragment further downstream of the $chr_1$ determinant on cosmid pDNA206 (Nies et al. 1989a) could not be found on contig 712, which contained the pMOL28-located *cnr* and $chr_1$ determinants, but was found on contig 660 (Fig. 1C). This DNA fragment contained a *rpoH* gene (317-amino-acid product) that encodes the only heat-shock sigma factor of strain CH34 (Fig. 1C). The *ropH* gene was successfully amplified from total DNA of the plasmid-free *R. metallidurans* strain AE104 (data not shown). Therefore, the *rpoH* gene was located on the bacterial chromosome and the *rpoH*-containing DNA fragment of cosmid pDNA206 did not originate from plasmid pMOL28.

Further analysis of the genomic sequence of *R. metallidurans* revealed the existence of a second *chr* determinant, $chr_2$ (DOE/JGI, Fig. 1B). Again, the $chr_2$ genes were successfully amplified from total DNA of the plasmid-free *R. metallidurans* strain AE104 (data not shown) indicating a chromosomal location of $chr_2$. This $chr_2$ determinant on contig 652 starts with $chrB_2$ ($ChrB_2$ 66% identical to $ChrB_1$), extends with $chrA_2$ ($ChrA_2$ 84% identical to the membrane protein $ChrA_1$) and ends with $chrF_2$ ($ChrF_2$ 76% identical to $ChrF_1$), all oriented in the same direction of transcription (Fig. 1B). Flanking these three genes were two ORFs probably not connected to chromate resistance. Both were located on the other DNA strand. One gene product was similar to a cytochrome P450 hydroxylase from *Bacillus halodurans* (gene 4756, BLAST score $8\times10^{-39}$), the other was similar to IciA (ORF 4754, BLAST score $3\times10^{-56}$), an inhibitor of chromosome replication initiation from *P. aeruginosa* (Fig. 1B).

Thus, in addition to the *rpoH* gene, a total of at least nine genes might be involved in chromate resistance of *R. metallidurans*: $chrB_2$, $chrA_2$, and $chrF_2$ as part of the chromosomal $chr_2$ determinant, and *chrI*, $chrB_1$, $chrA_1$, *chrC*, *chrE*, and $chrF_1$ as part of the $chr_1$ determinant on plasmid pMOL28. To characterize their functions, most of these genes were mutated and chromate resistance of the respective mutant strains was determined.

### Influence of the $chr_1$ genes on chromate resistance

Deletion of $chrA_1$ of the $chr_1$ determinant on plasmid pMOL28 led to a complete loss of the plasmid pMOL28-encoded chromate resistance as has already been described (Nies et al. 1990). To prevent polar effects when deleting $chrB_1$ and *chrC*, each of these genes was replaced by homologous recombination with a residual 60-bp ORF. Each ORF was in the first 27 and last 27 base pairs identical to the 5′ and 3′ ends of the respective target gene and contained the recognition sequence of a restriction endonuclease in the middle.

Chromate resistance of the resulting mutant strains was characterized in two different mineral salts media containing either 3 mM or 30 μM sodium sulfate as the sulfur source. This was done for two reasons. First, chromate is taken up into *R. metallidurans* cells by a sulfate uptake system. Therefore, chromate uptake into the cells is enhanced when the cells experience sulfate starvation. A concentration of 30 μM sulfate in the growth medium leads to induction of sulfate uptake systems (Nies and Silver 1989a). For sulfate-starved cells, chromate should be more toxic than for cells cultivated in high-sulfate conditions. This was indeed demonstrated for *R. metallidurans* strain AE126 (pMOL28) and its plasmid-free derivative strain AE104 (Table 1).

Second, at least 0.25 mM sulfate in the growth medium is required for efficient induction of the $chr_1$ resistance determinant (Peitzsch et al. 1998). Therefore, all experiments described below were done in high-sulfate (3 mM) and in low-sulfate (30 μM) medium.

Deletion of *chrC* resulted in a significant and reproducible loss of 1/7 or 1/8 of chromate resistance in the presence of both sulfate concentrations (Table 1). Deletion of *chrI* upstream of $chrB_1$ (Fig. 1A) led to a decrease in chromate resistance similar to the effect of the *chrC* deletion (Table 1). This showed that *chrI* and *chrC* might indeed be part of the $chr_1$ resistance determinant on plasmid pMOL28.

Deletion of $chrB_1$ from plasmid pMOL28 had no effect on chromate resistance (Table 1). However, the $chr_2$-encoded protein $ChrB_2$ might be able to substitute for the missing $ChrB_1$ protein. Therefore, the influence of the genes of the chromosomal $chr_2$ determinant on chromate resistance was also analyzed.

## Influence of the chromosomal $chr_2$ genes on chromate resistance

The chromosomal $chr_2$ genes and the *rpoH* gene were inactivated by insertional mutagenesis. Inactivation of *rpoH* had no effect on chromate resistance, even in the presence of plasmid pMOL28 (Table 1). Thus, the chromate resistance determinants in *R. metallidurans* were not or not exclusively transcribed by a RpoH-dependent RNA polymerase.

A $chrA_2$ mutation led to a loss of chromate resistance in high- and low-sulfate media (Table 1). The presence of each ChrA protein led to a duplication of chromate tolerance in the respective bacterial strain at either sulfate concentration (Table 1). Since $ChrA_2$ should be a chromate efflux pump, like its relative $ChrA_1$ (Nies et al. 1990, 1998), this indicates that both ChrA proteins might be involved in chromate detoxification in *R. metallidurans* cells.

Inactivation of $chrB_2$ also led to a complete loss of chromate resistance in both media (Table 1). This result was in contrast to that obtained following deletion of the $chrB_1$ gene on plasmid pMOL28, which had no effect on chromate resistance, indicating that $ChrB_2$ might indeed be able to substitute for a missing $ChrB_1$ protein. Thus, involvement of a ChrB protein in chromate resistance could be demonstrated.

The second $chr_2$ gene with a homologue in the $chr_1$ determinant was $chrF_2$ (Fig. 1). Inactivation of this gene led to increased resistance to chromate in high-sulfate medium, but had no effect in low-sulfate medium (Table 1). This indicates involvement of $ChrF_2$ in chromate resistance under certain environmental conditions.

The $ChrA_1$/$ChrA_2$ proteins should be chromate efflux pumps and the ChrC protein might scavenge superoxide radicals that originate when chromate interacts with cellular compounds (Liu et al. 1997). All three proteins might therefore be involved in direct detoxification of chromate. To investigate whether ChrI, ChrF and ChrB are regulators of chromate resistance, a reporter system for chromate-dependent regulatory processes was established. Transcripts of the $chr_1$ region were analyzed; the $chr_1$ promoters were identified and cloned as reporter gene fusions that were used to characterize the $chr_1$ regulatory genes.

## Transcription of the $chr_1$ determinant

The transcripts of the $chr_1$ region (Fig. 1A) were analyzed by Northern hybridization (Fig. 2). The signals of the $chrA_1$ and $chrB_1$ transcripts (Fig. 2A, B, respectively) started below the 23S rRNA (2,904 nucleotides). When scanned, the $chrA_1$ and $chrB_1$ signals both had a first maximum at a size of 2,420±330 nucleotides. A second maximum appeared at 1,300±100 nucleotides for $chrA_1$ or at 950±300 nucleotides for $chrB_1$ (Fig. 2, scanning data not shown). Both sizes matched the sizes of the respective genes (Fig. 1A). Since the 2,420-nucleotide message was sufficient to span the complete $chrBA_1$ region (Fig. 1A), $chrA_1$ and $chrB_1$ are likely to be transcribed as a dicistronic mRNA; however, additional monocistronic messages seem to occur. As observed with the reporter gene fusions (Peitzsch et al. 1998), transcription of $chrA_1$ and $chrB_1$ was induced by chromate. Transcription was increased when the cells were cultivated in the presence of high sulfate concentrations (3 mM) compared to cultivation under sulfate starvation (30 μM, Fig. 2).

The start sites of the $chrB_1$ and $chrA_1$ genes were determined by primer-extension (Table 2, Fig. 1A). The nucleotide sequences upstream of both start sites had some similarity to the consensus sequence CGnnnnnc TtGaa –13.5- cGCCc tnTA –3.5- tCCc. The start position of the $chrB_1$-mRNA, however, was only 4 bp away from the ATG start codon (Table 2) and was probably a truncated derivative of the original mRNA. Therefore, this consensus sequence may not indicate the correct position of the $chrBp_1$ promoter.

The *chrC* gene was transcribed as a 1030-nucleotide mRNA (Fig. 1A, Fig. 2D) that was sufficient in size to be a dicistronic *chrCE* transcript (Fig. 1A). Transcription of *chrC* was induced by chromate and was also better in high-sulfate- than in low-sulfate-grown cells. There was also a low level transcription of *chrC* in cells cultivated at 30 μM sulfate in the absence of chromate (Fig. 2D, lane 19).

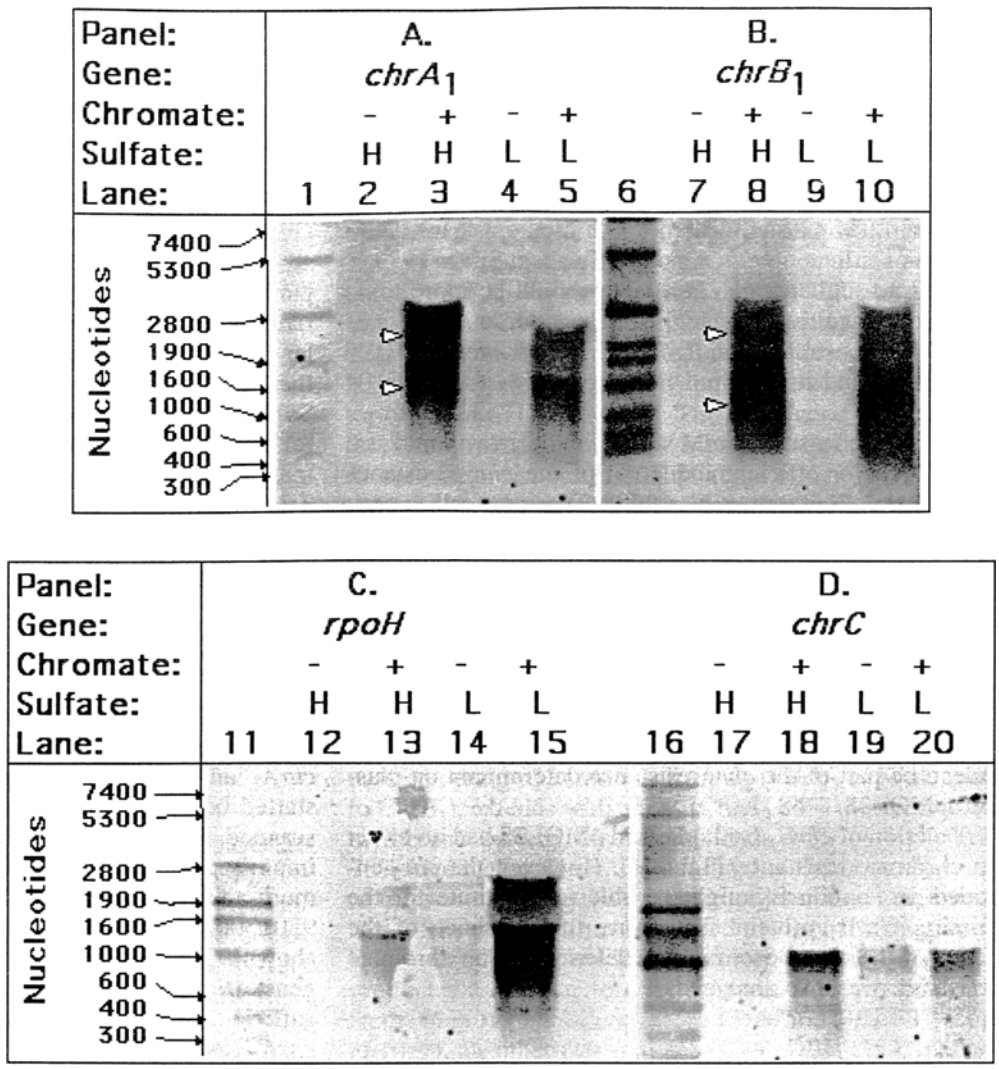


**Fig. 2** Transcripts of the $chr_1$ genes. *R. metallidurans* strain AE126(pMOL28) was cultivated in the presence of 3 mM sulfate (sulfate H) or 30 µM sulfate (sulfate L). The cells were induced for 10 min with 50 µM of chromate (chromate +) or not (chromate –). The RNA was isolated, Northern-blotted and probed with fluorescent-labeled probes for the genes $chrA_1$ (**A**), $chrB_1$ (**B**), *rpoH* (**C**) or *chrC* (**D**). The original gels were scanned and processed with Adobe Photoshop. The position of the size markers (*lanes A1, B6, C11, D16*) is given at the *left* side in nucleotides. *Open arrowheads* in **A**, **B** indicate the peak maxima of the scanned lanes at 2,420 nucleotides (*A3, B8*), 1,300 nucleotides (*A3*) and 950 nucleotides (*B8*)

**Table 2** Predicted promoter sequences and 5′ ends of the *chr* mRNAs. The 5′ ends of $chr_1$ mRNAs and the *rpoH*-specific message were measured by primer-extension analysis and are given in *bold-faced letters*. The predicted –35 and –10 regions are *underlined*. The nucleotide sequences up to the ATG start codons (*italic*) of the respective genes are given with the position of the last base indicated. *chrC* is the complete gene corresponding in its 5′ end to the previous *ORF3*. *Cons* Possible consensus motif. Numbers indicate the main distances between the parts of the motif given in base pairs

| Gene | DNA sequence in 5′→3′ orientation |
|---|---|
| $chrB_1$ | CTACAACA TTGAA GTAGCGCATACAG CCCGC TAAG CTG TCTGC *ATG* -0196 |
| $chrA_1$ | CAGTGGCT TTGAGGCAGTGCTGGCAGGCGCCC GGGA ACGCC **TCCCT**<br>AACGACGACGCACTGCTGGATGAAGTCGGCTATGTCCTCGACT<br>CGCTGTACACGCATTTCTCAAGCCCGCGCAAACGCTAGTCGTT<br>GCCAATCAACAAAGAAGCAAACTATTCAACAGACACG*ATG* -1212 |
| *chrC* | CGCGGTGA TCGTGTTGGCGAAGCGGT CGATC GTTG ATA TCCC<br>GACAGCGTTGCTGGCGCTTGTCACCGTCGCATTGCTTCTGAAGTTCA<br>AGAAGCTGTCGAGCCCATGATCGTTGCCGGGGCGGCCTTGATTGGT<br>CTGGTCGCCTATCCGTTGTTGCATCACTGAGATGCCACAGGAGACTCTT*ATG* -2437 |
| *rpoH* | CGATTGCC TGGAACTATCAACCCTATA GGCCT TCTA ACTT AGCA<br>CTCGTCCGGTTCGAGTGCTAATATGCGTTGCA*ATG* -6439 |
| Cons.: | CGnnnnnn TtGaa -13.5-cGCCc tnTA -3.5- tCCc |

Primer-extension experiments with *chrC* revealed a promoter, *chrCp*, upstream of the mRNA start site that showed some sequence similarity to the other two promoters, $chrBp_1$ and $chrAp_1$ (Table 2). All three sequences had some similarities to the consensus sequence for heat-shock sigma factors in *E. coli* (Cowing et al. 1985) and *Caulobacter* (Roberts et al. 1996; Wu and Newton 1996). Therefore, transcription of the *rpoH* gene was also studied (Fig. 2C). The *rpoH* gene was induced by chromate, but, in contrast to the *chr* genes, expression was stronger in low-sulfate than in high-sulfate medium (Fig. 2C). Although not essential for chromate resistance, this indicates that a possible influence of RpoH on chromate resistance may occur especially under conditions of sulfate starvation.

The *rpoH* mRNA signal had one maximum at 2,270 nucleotides (Fig. 2, scanning data not shown) and was much larger than the *rpoH* gene. However, either gene upstream and downstream of *rpoH* is on the other DNA strand and not connected to chromate resistance or its regulation. The promoter of *rpoH* was also similar to RpoH promoters (Table 2).

Taken together, the results show that regulation of the $chr_1$ resistance determinant occurs at the level of transcription initiation and is influenced by chromate and by sulfate. Surprisingly, transcription of the *rpoH* gene for a putative heat-shock sigma factor is also influenced by both anions.

**Table 3** Activity of *chr* promoters in mutant strains of *R. metallidurans*. Cells were grown in Tris-buffered mineral salts medium containing either 3 mM or 30 µM sulfate and 2 g sodium gluconate/l with shaking at 30 °C and the basal level of β-galactosidase activity from a $chrAp_1$::*lacZ* or a $chrBp_1$::*lacZ* fusion was determined. Half of the culture was induced with 50 µM chromate (3 mM sulfate) or 20 µM chromate (30 µM sulfate) and incubation was continued with shaking for 3 h at 30 °C. The chromate concentrations used were the optimum inducer concentration at the respective sulfate concentration (Fig. 3). β-Galactosidase activity was determined in the uninduced control and divided by the basal level expression leading to $IR_{unind}$. Similarly, β-galactosidase activity was determined in the induced cells and also divided by the basal level expression leading to $IR_{ind}$. The induction ratio $IR_{ind}$ was divided by the control ratio $IR_{unind}$. All points were done in triplicate in each experiment. Moreover, each experiment was done at least three times independently, and the standard deviations of these three experiments are shown. *nd* Not determined. Table W2 of the electronic supplementary material has an extended version of this table that contains the $IR_{ind}$, the $IR_{unind}$ and the basal level expression data

| Promoter | Genetic background | $IR_{ind}/IR_{unind}$ 50 µM $CrO_4^{2-}$ 3 mM $SO_4^{2-}$ | $IR_{ind}/IR_{unind}$ 20 µM $CrO_4^{2-}$ 30 µM $SO_4^{2-}$ |
|---|---|---|---|
| *chrAp* | pMOL28 | 0.91 | n. d. |
| | Δ*chrB*$_1$ | 0.91 | n. d. |
| | Δ*chrI* | 0.93 | n. d. |
| | No pMOL28 | 1.04 | n. d. |
| *chrBp* | pMOL28 | 4.88 | 1.90 |
| | Δ*chrB*$_1$ | 4.46 | 3.24 |
| | Δ*chrI* | 4.32 | 5.52 |
| | Δ*chrC* | 10.4 | 1.83 |
| | Δ*rpoH* | 15.1 | 9.42 |
| | No pMOL28 | 2.20 | 1.77 |
| | Δ*chrB*$_2$ | 1.02 | 1.03 |
| | Δ*chrF*$_2$ | 10.5 | 3.84 |

## Influence of the pMOL28-encoded chrI genes on expression from chr promoters

Information about the position of the promoters $chrBp_1$ and $chrAp_1$ was used to construct reporter systems for the identification of chromate-dependent regulators. Both promoters were cloned upstream of a promoterless *lacZ* gene on a broad-host-range plasmid with a low copy number. Activity from $chrAp_1$ was higher than activity from $chrBp_1$. However, expression of *lacZ* from $chrAp_1$ was never inducible by chromate (Table 3) and this promoter was therefore not further studied.

In the presence of plasmid pMOL28, expression of β-galactosidase activity from the $chrBp_1$::*lacZ* fusion was induced five-fold in high-sulfate medium (3 mM sulfate, 50 µM chromate, Table 3) and two-fold in low-sulfate medium (30 µM sulfate, 20 µM chromate). Thus, transcription initiation from $chrBp_1$ was stimulated by chromate, and the $chrBp_1$::*lacZ* fusion could be used to search for chromate-dependent regulators. In the absence of plasmid pMOL28, induction of the $chrBp_1$::*lacZ* fusion by chromate was about two-fold at both sulfate concentration (Table 3). Thus, the presence of plasmid pMOL28 significantly changed the effect of sulfate on chromate-dependent induction of the reporter construct. The effect of the

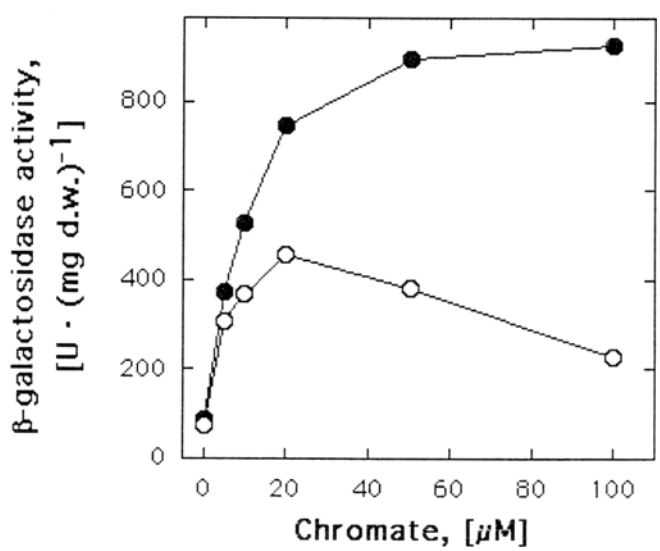


**Fig. 3** Activity of a *chrBp*::*lacZ* fusion in two different *R. metallidurans* strains. *R. metallidurans* strain AE126(pMOL28)(●) and the plasmid-free strain AE104 (○), both containing the plasmid pVDZ'2 derivative with a $chrBp_1$::*lacZ* transcriptional fusion, were cultivated at 30 °C with shaking in Tris-buffered minerals salts medium containing 3 mM sulfate and 2 g sodium gluconate/l. Various chromate concentrations were added ($t$=0), incubation with shaking was continued for 3 h, and β-galactosidase activity was determined

two different sulfate concentrations was very small in the absence of plasmid pMOL28 (1.2-fold), but increased in the presence of this plasmid (2.6-fold). The presence of plasmid pMOL28 enhanced chromate-dependent induction of the reporter fusion over a wide range of concentrations (Fig. 3). This indicates that genes located on plasmid pMOL28 might be required for the effect of sulfate on chromate-dependent induction of $chrBp_1$.

Deletion of $chrB_1$ or of *chrI* from plasmid pMOL28 had no effect on chromate-dependent induction of the $chrBp_1$*::lacZ* reporter in the high-sulfate medium, but led to increased induction in the low-sulfate medium (Table 3). The effect of the Δ*chrI* deletion was even stronger than the effect of the Δ$chrB_1$ deletion. This indicates that both gene products decreased chromate-dependent induction of $chr_1$ under low-sulfate conditions.

In contrast to the effect of the Δ$chrB_1$ and the Δ*chrI* deletions, deletion of *chrC* had no effect on chromate-dependent induction of the reporter in low-sulfate medium, but led to a ten-fold induction in high-sulfate medium (Table 3). If ChrC is indeed a SOD that repairs chromate-generated damage in the form of superoxide radicals, then a more efficient chromate detoxification by the ChrA efflux pumps is needed when ChrC is not present. However, this process seems to occur only under high-sulfate conditions.

## Influence of chromosomal genes on expression from $chrBp_1$

Inactivation of *rpoH* led to a strong increase in chromate-dependent induction of $chrBp_1$*::lacZ*. At either sulfate concentration the maximum expression level observed in all experiments occurred in the *rpoH* mutant background (Table 3). The cells seemed to have compensated for the absence of the heat-shock sigma factor RpoH with enhanced chromate detoxification by efflux.

Deletion of the $chrB_2$ gene completely abolished induction of $chrBp_1$ by chromate under both sulfate conditions (Table 3). Thus, $ChrB_2$ was identified as an essential activator of chromate-dependent induction of $chrBp_1$, which explains also the essential function of $ChrB_2$ for chromate resistance in the pMOL28-free strain AE104 (Table 1). Finally, deletion of $chrF_2$ led to a strong increase in induction under both sulfate concentrations. Thus, ChrF might be a repressor for chromate-dependent induction of $chrBp_1$. Furthermore, ChrB and ChrF are the first identified regulators of an inducible chromate resistance determinant.

## The C-terminus of $ChrB_1$ did not serve as a leader sequence for export of $ChrB_1$ into the periplasm

$ChrB_1$ contains a hydrophobic amino acid stretch at the amino terminus, which is flanked by a negatively charged Glu residue on one side and four positively charged amino acid residues on the other (9-ETAWLLLVVSLPTSAS-TARMRFWRGIK-35, apolar cluster underlined). This

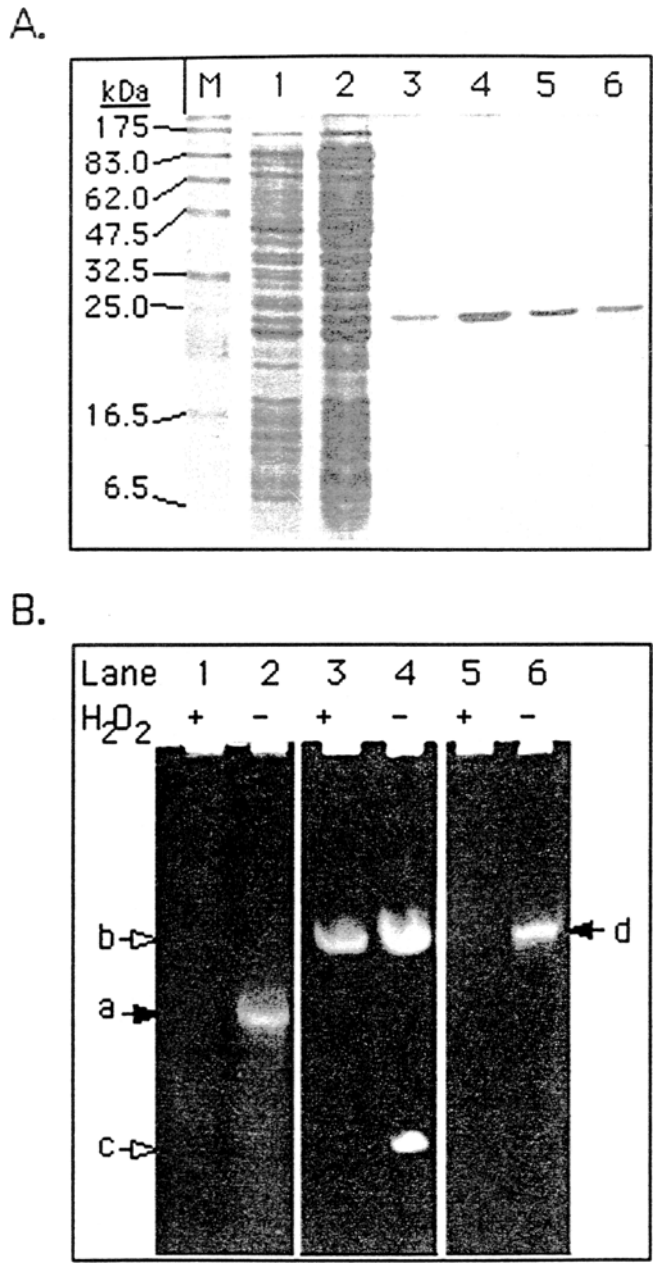


**Fig. 4** ChrC is a superoxide dismutase (SOD). The ChrC protein was expressed as a N-terminal Strep-TagII fusion in *Escherichia coli* and purified by affinity chromatography (**A**). The Coomassie-stained SDS-polyacrylamide gel shows the purification of ChrC. Crude extract of *E. coli* cells before (*lane 1*) and after (*lane 2*) induction with anhydrotetracycline and elution fractions 1–4 of the affinity chromatography (*lanes 3–6*) are shown. *M* Size-marker lane with the sizes given to the *left*. The purified ChrC protein and controls were applied to a 8% native polyacrylamide gel and the gel was stained for SOD activity (**B**). Crude extracts (50 µg per lane) from cells of *R. metallidurans* strain AE126(pMOL28) (*lanes 1, 2*), from cells of *E. coli* (*lanes 3, 4*), and purified ChrC protein (2.5 µg per lanes; *lanes 5, 6*) were treated with 0.5 g $H_2O_2$/l (*lanes 1, 3, 5*) or without $H_2O_2$ (*lanes 2, 4, 6*), separated on a polyacrylamide gel, and stained for SOD-specific activity. *Arrows* SODs from *R. metallidurans* (*a*), the Mn-SOD from *E. coli* (*b, open arrowhead, middle lanes*), the Fe-SOD from *E. coli* (*c, open arrowhead, middle lanes*), and ChrC (*d*)

could be a leader sequence. A *chrB*$_1$′::*phoA* fusion was constructed at position $M_{75}$ and two independent clones were expressed in *E. coli*. The specific activities of the fusion proteins in the two strains were 0.73±0.11 U (mg dry weight)$^{-1}$ and 0.72±0.04 U (mg dry weight)$^{-1}$. These values were comparable to 0.53±0.02 U (mg dry weight)$^{-1}$, the value determined for a negative fusion control that carries a leader-free β-lactamase gene. The positive control, which contains the respective leader peptide, had a specific activity of 6.58±0.59 U (mg dry weight)$^{-1}$. Thus, the PhoA-domain of the ChrB$_1$′::PhoA hybrid protein was not exported into the periplasm and ChrB$_1$ may not be a periplasmic protein.

### The *chrC* gene encodes a superoxide dismutase

The predicted ChrC protein showed sequence similarities to SODs with manganese or iron in the active center. To investigate whether ChrC is indeed a SOD, the protein was expressed as a N-terminal Strep-TagII fusion and purified by affinity chromatography as a 24-kDa fusion protein (Fig. 4A). Analytical ultracentrifugation indicated an apparent molecular mass of 98 kDa for the native protein (data not shown). Thus, ChrC is probably a homotetrameric protein. The ChrC protein was an active SOD as judged by the result of an activity stain (Fig. 4B, lane 6).

A SOD from *R. metallidurans* was recently purified by Roux and Coves (Roux and Coves 2002). This SOD was the only superoxide dismutase visible in crude extracts of this bacterium and migrated faster than ChrC in a polyacryamide gel (Fig. 4B). No signal was visible in crude extracts of *R. metallidurans* at the position of ChrC, neither in untreated cells (Fig. 4B) nor after induction of *chr* with 50 μM chromate (data not shown). As described (Roux and Coves 2002), the Fe-SODs from *R. metallidurans* and *E. coli*, but not the Mn-SOD from *E. coli*, could be inhibited by $H_2O_2$ (Fig. 4B). Since ChrC could also be inhibited by $H_2O_2$ (Fig. 4B lane 5), this indicates that ChrC is more likely a Fe-SOD than a Mn-SOD. In agreement with this conclusion, ChrC exhibited an iron signal, but not a manganese signal, in the EDX spectrum (data not shown). Therefore, ChrC is probably a tetrameric, Fe-containing SOD.

## Discussion

### Chromate resistance in *R. metallidurans*

Chromate enters the cell of *R. metallidurans* by (the) sulfate uptake system(s), and sulfate starvation (30 μM instead of 3 mM sulfate in the growth medium) leads to tenfold enhanced chromate accumulation (Nies and Silver 1989a). In the presence of 3 mM sulfate, sulfate uptake is repressed. Furthermore, sulfate inhibits chromate uptake competitively (Nies and Silver 1989a). In agreement with these data, the minimal inhibitory concentration (MIC) of chromate, as an overall value of all strains tested (Table 1), was about eight-fold higher in high-sulfate medium than in low-sulfate medium. The ratio of the lowest MIC determined for all mutant strains and both sulfate concentrations (10 μM, Δ*chrA2* mutant derivative of strain AE104) and the highest MIC (Table 1) was only 35-fold. This value of 35 is the arithmetical product of eight (the effect of the sulfate concentrations) and four to five (the effect of the presence of both chromate resistance determinants, Table 1). Thus, the major part of chromate resistance in *R. metallidurans* can be contributed to decreased uptake of chromate as the result of repression of the sulfate uptake system(s).

There are now two known determinants involved in chromate detoxification in *R. metallidurans*. One (*chr*$_2$) is located on the bacterial chromosome, the other (*chr*$_1$) on megaplasmid pMOL28. Both *chr* determinants should detoxify the oxyanion mainly by efflux, resulting in a decreased accumulation of chromate in the cell (Cervantes et al. 1990; Nies et al. 1990; Alvarez et al. 1999; Pimentel et al. 2002). Each *chr* determinant increased chromate resistance just two-fold, whereas in the presence of both determinants resistance increased four-fold in low-sulfate medium and five-fold in high-sulfate medium. These are small effects compared to other metal-resistance systems, some of which increase the MIC values by a factor of more than 100 (Nies et al. 1989b; Rensing et al. 1997). One explanation for this difference may be that cytoplasmic chromate escapes the ChrA chromate efflux pumps. Rapid interaction of chromate with some cellular components or chromate reduction (Peitzsch et al. 1998) could lead to toxic effects of the oxyanion that are not compensated for by chromate efflux (Cervantes et al. 1990; Nies et al. 1990; Alvarez et al. 1999).

Chromate-induced expression of the putative heat-shock sigma factor RpoH was greater in low-sulfate than in high-sulfate medium. This was in contrast to expression of ChrA$_1$, ChrB$_1$ and ChrC. Deletion of *rpoH* yielded a clear effect on chromate-dependent induction of a *chr*$_1$::*lacZ* reporter fusion. The deletion strain exhibited a very high expression level of the reporter fusion after chromate induction. Since this mutant strain showed no decreased chromate resistance (Table 1) and was able to initiate transcription from *chrBp*$_1$, RpoH is not essential for expression of the *chr* determinants. However, products of genes expressed under RpoH control may contribute to chromate detoxification, similar to defense against other stressors (Bukau 1993; van Dyk et al. 1995; Gross 1996; Manzarena et al. 2001). These gene products could repair damage done by chromate that has escaped detoxification by the ChrA efflux pumps.

Another possible repair function is exerted by ChrC. ChrC is a superoxide dismutase and probably a tertrameric protein. As the EDX spectrum and the inhibition of its activity by $H_2O_2$ showed, ChrC is more likely to be of the Fe-type than of the Mn-type. However, even after induction with chromate, activity of this enzyme was not visible on activity-stained polyacrylamide gels (Fig. 4), since the specific activity of ChrC was only about 1% of that of the Fe-SOD purified by Roux and Coves [(2002),

data not shown]. Thus, in the presence of chromate, *R. metallidurans* cells seem to produce the low-activity Fe-SOD ChrC in addition to the main cellular Fe-SOD. The reason for this may be that ChrC does not detoxify superoxide radicals, but has another unknown function.

The ORF following *chrC* on plasmid pMOL28 was named *chrE* because it is located between the $chr_1$ genes *chrC* and $chrF_1$ and may be transcribed together with *chrC*. ChrE was 22% identical to a putative protein from *P. aeruginosa*, PA0589. Both proteins show sequence similarity to a rhodanese in the BLAST search (data not shown). A rhodanese-type enzyme has been proposed to clear off seleno-glutathione-complexes that are formed by the reaction of selenite with glutathione (Self et al. 2000). Since chromate interacts with glutathione (Arslan et al. 1987; Debetto et al. 1988), ChrE may cleave some chromium-glutathione-complexes.

## Four proteins form the core regulatory system of chromate detoxification in *R. metallidurans*

Deletion of the genes $chrB_2$ and $chrF_2$ could identify the proteins encoded by these genes as *chr* regulators only in the absence of megaplasmid pMOL28. The amino acid sequence of ChrB1 was 66% identical to that of $ChrB_2$ and should have a similar function as an essential chromate-dependent activator. The 136-amino-acid carboxy-terminus of $ChrB_2$ (starting at W-200) and $ChrF_2$ (152 amino acids) also showed 30% identity. Similarly, the C-terminus of $ChrB_1$ (starting at QW-189) was 32% identical to $ChrF_2$. $ChrF_1$ and $ChrF_2$ were 76% identical (Fig. 1). All these proteins from *R. metallidurans* were related to putative proteins (Fig. W1, electronic supplementary material) from genome sequencing projects, indicating the presence of ChrF- and ChrB-like proteins in other bacteria. Both proteins also occurred in *Ralstonia solanacearum*, a plant pathogen related to *R. metallidurans* (Goris et al. 2001). In contrast to *R.* metallidurans, *R. solanacearum* contained only one copy of either gene. The closeness of the relationship of the three respective ChrF, ChrB (Fig. W1, electronic supplementary material) and ChrA proteins (data not shown) from the two *Ralstonia* species is striking. Duplication of the two sets of chromate resistance genes in *R. metallidurans* may have occurred during speciation of the genus *Ralstonia*, perhaps indicating an adaptation of *R. metallidurans* to environments with elevated chromate concentrations, e.g. serpentine soils (Baker 1987).

The four $ChrB_1$-related proteins may form a core regulatory system for chromate-dependent *chr* regulation in *R. metallidurans*. This core system is composed of the larger, possibly membrane-bound ChrB-type activators and the smaller ChrF-type repressors. The sequence similarity of the four proteins, their possible location, and their function in regulation of chromate resistance points to the involvement of a signal transduction system in chromate-dependent regulation of chromate resistance.

## The pMOL28-encoded $chr_1$ determinant seems to detoxify chromate especially under high-sulfate conditions

$ChrB_1$ repressed $chrBp_1$ under low-sulfate conditions, but not under high-sulfate (Table 3). This effect is even more pronounced when another $chr_1$-reporter fusion was analyzed: expression was four-fold higher under high-sulfate medium than under low-sulfate, but only in the absence of megaplasmid pMOL28 (Peitzsch et al. 1998). This indicates that the function of the pMOL28-encoded $chr_1$ determinant may be to detoxify chromate especially at high-sulfate concentrations.

The difference between the plasmid- and chromosomally encoded *chr* determinants is the presence of *chrI, chrC* and *chrE* genes on megaplasmid pMOL28. ChrI is related to two proteins with unknown functions from *P. syringae* (AF359557) and *P. aeruginosa* (AE004947). The ChrI protein repressed induction of $chrBp_1$ at low-sulfate concentrations similar to $ChrB_1$, additional evidence that $chr_1$ encodes high-sulfate chromate resistance. Affiliation of ChrE with the rhodanese superfamily (Bordo and Bork 2002) also suggests a relationship with sulfur metabolism. Finally, the absence of ChrC led to enhanced expression of $chr_1$ (Table 3), but exclusively under high-sulfate conditions. Thus, the two chromate resistance determinants from *R. metallidurans* may complement each other under different ecological conditions, allowing the bacterium to grow despite the presence of varying sulfate and chromate concentrations.

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