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ORIGINAL PAPER

Characterization of two genes involved in chromate resistance in a Cr(VI)-hyper-resistant bacterium

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Abstract Mechanisms underlying chromate resistance in Cr(VI)-hyper-resistant *Pseudomonas corrugata* strain 28, isolated from a highly Cr(VI) polluted soil, were studied by analyzing its two Cr(VI)-sensitive mutants obtained by insertion mutagenesis. The mutants, namely Crg3 and Crg96, were characterized by the identification of disrupted genes, and by the high-throughput approach called Phenotype MicroArray (PM), which permitted the assay of 1,536 phenotypes simultaneously. Crg3 and Crg96 mutants were affected in a malic enzyme family gene and in a gene encoding for a RecG helicase, respectively. The application of PM provided a wealth of new information relating to the disrupted genes and permitted to establish that chromate resistance in *P. corrugata* strain 28 also depends on supply on NADPH required in repairing damage induced by chromate and on DNA integrity maintenance.

Keywords *Pseudomonas* · Cr(VI)-resistance · Phenotype MicroArray · RecG helicase · Malic enzyme

Introduction

Hexavalent chromium is a severe contaminant since it, differently from trivalent chromium, is highly water soluble and toxic, mutagenic to most organisms, and carcinogenic for humans (Viti and Giovannetti 2007). A comprehensive understanding of bacteria Cr(VI)-toxicity and bacterial Cr(VI)-resistance has yet to be achieved, even though significant advances have been gained (Ramirez-Diaz et al. 2008). Studies made by high-throughput genomics and proteomics approaches have brought to light some Cr(VI)-resistance mechanisms (Hu et al. 2005; Brown et al. 2006). These technologies allow for global analysis of the important macromolecules of cells that convey the information flow from DNA to RNA to protein. However, the information initially encoded in the genome is ultimately displayed at the cellular level as cellular traits or phenotypes. Therefore, combining reverse genetics and Phenotype MicroArray analysis (PM-Biolog), a high-throughput phenomic technology that permits to test nearly 2,000 phenotypes simultaneously, can greatly advance the understanding of Cr(VI)-resistance and -reduction in bacteria. This combined approach has been recently used to characterize a Cr(VI)-sensitive mutant and to understand the function of an impaired gene in Cr(VI)-resistance and sulfur metabolism (Viti et al. 2009).

Here, two genes involved in chromate resistance in a Cr(VI)-hyper-resistant (40 mM K_2CrO_4) isolate belonging to *Pseudomonas corrugata* (Viti et al. 2006) were identified by characterizing two Cr(VI)-sensitive mutants. The phenotypes associated with impaired genes were evaluated by the PM high-throughput analysis.

Materials and methods

Bacteria and growth conditions

Pseudomonas corrugata strain 28 [isolated from a soil highly polluted with Cr(VI)], resistant up to 40 mM of K_2CrO_4 (Viti et al. 2006) and deeply characterized (Viti

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et al. 2007), and its two Cr(VI)-sensitive mutants, namely Crg3 and Crg96, were used. The mutants were obtained by EZ-Tn5^{TM} <R6Kγ*ori*/KAN-2>Tnp Transposome mutagenesis kit (Epicentre Technologies) (Viti et al. 2009). To obtain mutants, a 40-µl aliquot of electrocompetent cells was mixed with 20 ng of transposome DNA (EZ-Tn5^{TM} <R6Kγori/KAN-2>Tnp Transposome Mutagenesis Kit), and then electroporation was performed with a Gene Pulser Xcell electroporator (Bio-Rad Laboratories) in 0.1-cm cuvettes at 2.0 kV, 125 µF, and 200 Ω. Cells were resuspended in 2 ml of SOC medium (Sambrook et al. 1989) and incubated at 30°C for 1 h. Transformants were selected onto TMM plates containing 15 µg kanamycin ml^{-1} (TMM/Km). Colonies, which reached a diameter of 2–3 mm after 3–5 days of incubation at 25°C, were picked and plated onto TMM/Km added with 20 mM K_2CrO_4 and then incubated at 25°C for 2 days. From screening of over 14,000 transformants, eight clones showing Cr(VI)-MICs lower than 20 mM were found. After susceptibility tests in liquid media, two stable Cr(VI)-sensitive mutants, Crg3 and Crg96, were selected.

Strains were grown on LB medium at 25°C in aerobic condition. For Cr(VI) susceptibility tests tris-minimal medium (TMM) (Mergeay 1995), added with 0.2% Na-gluconate (w/v) and different concentrations of K_2CrO_4 (Merck), was used.

DNA hybridization

To verify the single insertion of the EZ-Tn5^{TM} transposon in the genomes of the two mutants, Southern hybridization experiments were carried out as reported by Viti et al. (2009). DNA was isolated and digested with either *Pvu*II or *Mlu*I, which do not cut within EZ-Tn5^{TM} transposon. Electrophoretic separation and Southern hybridization were performed as described by Sambrook et al. (1989). The probe used was a fragment of kanamycin resistance gene of EZ-Tn5^{TM} transposon labeled with fluorescein by Gene Images random prime labeling kit (Amersham Life Sciences). Hybridization was performed at 55°C; the plot was developed using ECF detection system (Amersham Life Sciences), and subsequently scanned by a Typhoon 9200 (Amersham Life Sciences).

DNA sequencing and identification of the transposon insertion site

DNA extraction and cloning procedures were performed as described by Viti et al. (2009). Plasmid DNA was extracted from transformants (MiniElute kit, Qiagen) and bidirectionally sequenced using primers KAN-2 FP-1 and R6KAN-2 RP-1 provided with the EZ-Tn5 <R6Kγori/KAN-2>Tnp Transposome Mutagenesis kit (Epicentre Technologies). Sequencing was performed using BigDye Terminator Cycle Sequencing Kit on an ABI 3100 DNA sequencer (Applied Biosystems). DNA sequences obtained were viewed, edited with Chromas Lite (Technelysium Pty Ltd.) and aligned with ClustalX (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). Sequence analysis was performed with BlastN, and/or tBlastX, and/or BlastP programs in GenBank database (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov).

Chromate-minimum inhibitory concentration [Cr(VI)-MIC] and chromate reduction

Cr(VI)-MIC for each strain was determined in accordance with Viti et al. (2003). Cr(VI) reduction capability was investigated under aerobic conditions, using colorimetric diphenylcarbazide (DPC) method (Shen and Wang 1995). The cultures were incubated at 25°C under shaking conditions (200 rpm) until they reached stationary phase of growth.

Phenotype MicroArray (PM)

The strains were tested by the PM approach (PM01–PM04 and PM09–PM20) for 1,536 different conditions, including carbon, nitrogen, phosphorous and sulfur sources, several concentrations of ions and osmolites, a wide variety of antibiotics, antimetabolites, heavy metals and other inhibitors, and pH stress, using the procedures described by Viti et al. (2009). Assays of C (190 carbon sources), N (95 nitrogen sources), P (59 phosphorus sources), and S (35 sulfur sources) metabolism were performed because they provide information about which metabolic pathways are present and active in the cells (wild-type vs. mutants). The set of the chemical sensitivity assays includes chemicals that are toxic to most microorganisms by interfering with diverse cellular pathways (i.e., cell wall synthesis, cell membrane synthesis, nutrient transport, DNA replication, etc.). Such assays with those of cation (Na^+, K^+, Cu^{2+}, Fe^{3+}, Zn^{2+}, Mn^{2+}, etc.), anion (chloride, sulfate, chromate, phosphate, vanadate, nitrate, selenite, tellurite, etc.) and pH sensitivities provide information on stress and repair pathways that are present and active in cells (wild-type vs. mutants).

PM technology uses tetrazolium violet reduction as a reporter of active metabolism (Bochner et al. 2001). The reduction of the dye causes the formation of a purple color, which is recorded by a CCD camera every 15 min for 48 h, and provides quantitative and kinetic information about the response of the cells in the PM plates (Bochner et al. 2001). The data obtained are stored in computer files and can be analyzed to compare the PM kinetics of different strains (Koo et al. 2004; Winterberg et al. 2005). Each strain was

analyzed in duplicate, and a consensus result was obtained. To identify gained or lost phenotypes by each mutant with respect to strain 28, obtained kinetic curves were compared by Omnilog-PM software (release OM_PM_109M).

Results

Characterization of chromate-sensitive mutants

In order to deepen the knowledge about molecular mechanisms involved in chromate resistance in strain 28 (*P. corrugata*), which shows a Cr(VI)-MIC of 40 mM, two stable Cr(VI)-sensitive mutants, obtained by transposon mutagenesis insertion and named Crg3 and Crg96, were used.

To verify the single insertion of EZ-Tn5^{TM} transposon in the genome of the mutants, Southern hybridization experiments with a fragment of the kanamycin resistance gene of the EZ-Tn5^{TM} transposon as a probe were attempted. The results obtained showed a single hybridized band in the two mutants, revealing that the EZ-Tn5^{TM} transposon insertion occurred only one time for each mutant at different genomic sites (data not shown).

Chromate-susceptibility test showed that Crg96 and Crg3 were strongly affected by chromate when compared to strain 28. Their Cr(VI)-MICs were 3 and 10 mM, respectively (Fig. 1). The Cr(VI)-reducing activity, measured as a decline in the concentration of hexavalent chromium in the cultivation medium, was lower in Crg3 and Crg96 mutants than in the wild-type strain (17.6 ± 1.4, 41.6 ± 1.1 and 52.3 ± 6.1%, respectively, with reference to the original chromate concentration in the medium 0.2 mM K_2CrO_4).

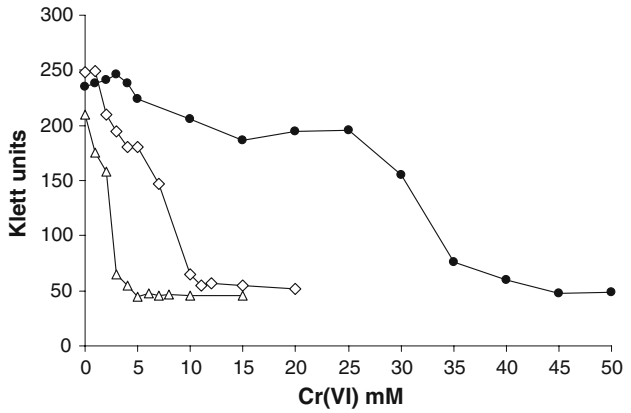

Fig. 1 Susceptibility to chromate of wild-type and mutants (*filled circle* wild-type; *open diamond* crg3; *open triangle* crg96). Cultures were grown in TMM added with 0.2% Na-gluconate at 25°C with shaking for 48 h with the indicated concentration of chromate, and the cell growth was measured as Kett units. *Data points* represent the average of three experiments with standard deviation less than 10% of given values

To identify the affected genes in the mutants, the DNA regions flanking the transposon insertion were sequenced, and data obtained were compared with known sequences in GenBank database. The nucleotide sequence disrupted by EZ-Tn5^{TM} insertion in Crg3 (GenBank accession number EU276968) had a high identity percentage to a gene encoding a protein (90% identity with *P. fluorescens* Pf-5, PFL_0443), which catalyzes the oxidative decarboxylation of L-malate to pyruvate with concomitant reduction of the cofactor NAD^+ or $NADP^+$, belonging to the malic enzyme family (ME). The nucleotide sequence disrupted by EZ-Tn5^{TM} insertion in Crg96 (GenBank accession number EU276971) had a significant identity percentage to the sequence of *recG* encoding the ATP-dependent RecG DNA helicase (83% identity with *P. fluorescens* Pf-5 PFL_6071), which plays a critical role in DNA recombination and repair (McGlynn and Lloyd 2001).

PM characterization of strain 28 and Crg mutants

Strain 28 and Crg mutants were phenotypically characterized by the innovative high-throughput technology Phenotypic MicroArray (Viti et al. 2007). Metabolic panels (PM01-PM04, PM09-PM10), and sensitivity panels (PM11-PM20) were used to monitor 1,536 phenotypic characters (detailed information about PM panels is available at http://www.biolog.com).

The phenotypes gained or lost by each Crg mutant with respect to strain 28 were identified analyzing the consensus data obtained from two independent experiments (Table 1). The comparison of kinetics curves obtained on PM01 and PM02 panels showed that Crg3 had a lower metabolic activity on D,L-malic acid, L-malic acid and D-saccharic acid, and an higher metabolic activity on D-mannitol.

No differences were found between strain 28 and Crg mutants regarding nitrogen, phosphorous, and sulfur compounds utilization (PM03 and PM04). Only the activity on L-guanine was higher in the mutant Crg96 than in the wild-type strain. A good deal of work will be required to establish any role for the impaired gene in the mutant and L-guanine metabolism.

Sensitivity panels (PM11–PM20) allowed to perform 1,152 different tests. As expected, the mutants, which harbor the kanamycin resistance gene of EZ-Tn5^{TM} transposon, gained resistance to kanamycin and to other aminoglycoside antibiotics. With respect to strain 28, Crg3 showed an increased sensitivity to two quinolone antibacterials (norfloxacin and pipemidic acid) that inhibit DNA topoisomerases, to 2-nitroimidazole and to some compounds inducing membrane damage (protamine sulfate, cetylpyridinium chloride, polymyxin B), to two fungicides, dodine and captan, that

Table 1 Phenotype of Crg mutants

Tests	Substrate/chemical	Mode of action	Phenotype of Crg mutants[a]	
			Crg3	Crg96
Metabolism (PM01–PM04 and PM09–PM10)				
Phenotypes gained: faster growth/resistance	D-mannitol	Carbon source	+89	
	L-guanine	Nitrogen source		+155
Phenotypes lost: slower growth/sensitivity	D-saccharic acid	Carbon source	−121	
	D,L-malic acid	Carbon source	−131	
	L-malic acid	Carbon source	−81	
Chemical sensitivity (PM11–PM20)				
Phenotypes gained: faster growth/resistance	2,2′-dipyridyl	Chelator, Fe^{2+}	+399	
	5-fluorouracil	Nucleic acid analog, pyrimidine	+234	
Phenotypes lost: slower growth/sensitivity	Norfloxacin	DNA topoisomerase, quinolone	−112	−103
	Pipemidic acid	DNA unwinding, gyrase (GN), topoisomerase (GP), quinolone	−282	−123
	Dodine	Fungicide, guanidine, membrane permeability	−304	
	Captan	Fungicide, carbamate, multisite	−589	−661
	Enoxacin	DNA topoisomerase, quinolone		−105
	Nalidixic acid	DNA topoisomerase		−129
	Rifampicin	RNA polymerase		−158
	Potassium chromate	Toxic anion	−109	−100
	Chromium chloride	Transport, toxic cation		−457
	L-glutamic-γ-hydroxamate	tRNA synthetase		−108
	Ciprofloxacin	DNA topoisomerase, quinolone		−307
	Tolylfluanid	Fungicide, phenylsulphamide		−535
	2-nitroimidazole	Ribonucleotide DP reductase	−314	
	Protamine sulfate	Membrane, ATPase	−192	
	Cetylpyridinium chloride	Membrane, detergent, cationic	−226	
	Polymyxin B	Membrane, outer	−127	

[a] The units are arbitrary. The OmniLog-PM software generates time course curves for respiration (tetrazolium color formation) and calculates differences in the areas for a mutant and wild-type strain. *Positive values* indicate that the mutant showed greater rates of respiration than the wild-type strain. *Negative values* indicate that the wild-type strain greater rates of respiration than the mutant. The differences are averages of values reported for two replicates of each mutant compared with the wild-type strain

are cytotoxic, and to the potassium chromate (Table 1). Also in Crg96 the main phenotypes lost were related to the resistance to quinolone antibacterials (norfloxacin, pipemidic acid, enoxacin, nalidixic acid, ciprofloxacin), and to the potassium chromate (Table 1). Furthermore Crg96 showed an increased sensitivity to the fungicides captan and tolylfluanid, to the chromium chloride, to the rifampicin (RNA polymerase inhibitor), and to the L-glutamic-γ-hydroxamate (t-RNA synthetase inhibitor) with respect to strain 28 (Table 1).

Discussion

This study was undertaken to provide new insights into basic bacterial cellular processes related to chromate stress and the mechanisms involved to counteract it. We identified two genes related to Cr(VI)-resistance in the Cr(VI)-hyper-resistant *P. corrugata* 28, and we tried to establish the links between function of impaired genes, by insertion mutagenesis events, and phenotypes applying PM approach.

In Crg3 EZ-Tn5 insertion occurred in ME encoding gene. PM results confirmed the inactivation of ME. Crg3 showed, with respect to strain 28, a lower activity on D,L-malic acid, L-malic acid, and on D-saccharic acid. The latter can be transformed into 2-oxoglutarate in *Pseudomonas* (Dagley and Trudgill 1965; Lerondel et al. 2006) and then into L-malic acid by enzymes of the tricarboxylic acid cycle. ME converts malate to pyruvate and CO_2 with the simultaneous generation of reducing power as NAD(P)H

(Driscoll and Finan 1996), and thus belong to a set of reactions that link the main pathways, which regulate the carbon flux between catabolism and anabolism, and the energy needs of cells. The lowest capability of this mutant to resist and reduce Cr(VI) could be explained with the lower capability of Crg3 to generate reducing power, since NAD(P)H-dependent Cr(VI)-reductases are present in several bacteria (Ishibashi et al. 1990; Suzuki et al. 1992; Park et al. 2000). The capability of Cr(VI)-reduction is suggested as an additional chromosome or plasmid resistance mechanism, and represents a potentially useful detoxification process for several bacteria (Komori et al. 1989; Cervantes et al. 2001; Pattanapipitpaisal et al. 2001). Park et al. (2000) isolated and characterized a dimeric flavin mononucleotide-binding flavoprotein (ChrR) that is able to catalyze a full reduction of Cr(VI) to Cr(III) and may contribute to withstand the unavoidable production of H_2O_2 that accompanies chromate reduction (Gonzalez et al. 2005).

It is well established that ME has a role in providing reducing power for repairing cellular damages due to oxidative stress in plants (de Aragao et al. 1997; Casati and Andreo 2001; Smeets et al. 2005) and in mammalian cells (Ayala et al. 1986; Revilla et al. 1987), but only little evidence is reported in bacteria (Singh et al. 2007). Our results suggest that in strain 28 ME has an important role in providing reducing power for repairing cellular damage due to chromate oxidative stress, since Crg3 showed a higher sensitivity than strain 28 to several compounds that induce oxidative stress such as norfloxacin, pipemidic acid, captan and 2-nitroimidazole (Yoshida et al. 1993; Kovacic and Osuna 2000; Suzuki et al. 2004; Dwyer et al. 2007), or membrane damage such as dodine, cetylpyridinium chloride, polymixin B and protamine sulfate (Cabral 1991; Johansen et al. 1997; Zhang et al. 2000).

PM results showed that Crg3 had a higher activity on D-mannitol with respect to strain 28. Mannitol is a widely distributed sugar alcohol in organisms (from bacteria to algae, fungi and more than 100 species of higher plants); moreover, it is considered a hydroxyl radical scavenger in vitro (Smirnoff and Cumbes 1989). The protective role of mannitol against toxic oxygen intermediates has been shown also in vivo in transgenic tobacco with a bacterial mannitol-1-phosphate dehydrogenase gene. Mannitol concentration was higher in transgenic plants than in non-transgenic plants; the former were more resistant to methyl viologen, a hydroxyl radical producing compound, than the latter (Shen et al. 1997). A similar result has been obtained by transforming *Saccharomyces cerevisiae* with a multicopy plasmid harboring a gene encoding the mannitol-1-phosphate dehydrogenase of *Escherichia coli*; the transformed strain produced mannitol and showed a higher resistance than the wild-type to oxidants (Chaturvedi et al. 1996). Thereby, the higher activity of Crg3 on D-mannitol might be due to the need of counteract hydroxyl radical activity associated with the growth.

Crg96 was defective in the *recG* gene encoding a RecG DNA helicase. This report is not the first indicating that RecG helicase is involved in Cr(VI) resistance (Miranda et al. 2005). RecG, whose function is to rescue stalled replication forks during DNA replication process (McGlynn and Lloyd 2001), is a really conserved enzyme present in Bacteria, Archaea, and Eukaryota. Chromosomal replication rarely progresses unimpeded from origin to terminator (Cox et al. 2000), and it is likely that replication forks stall at some points during the replicative process as a result of encountering various forms of lesion [polymerase arresting lesions (PALs)] in the DNA template. A major pathway for dealing with blocked replication forks involves RecG helicase, which catalyzes formation of Holliday junction via regression of the fork (McGlynn and Lloyd 2001). The Holliday junction formed by this process may then be migrated, for example by the RuvAB proteins, to re-establish the replication fork with the DNA lesion bypassed, for later repair.

PM characterization showed that Crg96 was much sensitive than 28 strain to genotoxic compounds, known to induce inhibition of DNA replication, such as quinolone antibiotics (norfloxacin, pipemidic acid, enoxacin, nalidixic acid, and ciprofloxacin), captan (Snyder 2006), and Cr(III). Quinolone antibiotics were shown to induce PALs, promoting the formation of a covalent link between DNA strands and enzymes gyrase or topoisomerase I, which block the replication fork (Goss et al. 1965; Wentzell and Maxwell 2000). Genotoxic activity of Cr(III) was extensively proved in vitro, but little evidence in vivo was reported. In vitro Cr(III) causes several kinds of DNA lesions (Köster and Beyersmann 1985; Arakawa et al. 2000), and PALs due to chromium-mediated DNA–DNA interstrand crosslinks (ICLs) (Bridgewater et al. 1994). Recently, experiments conducted in vivo on *E. coli* showed that chromium chloride induces several stress promoters associated with different types of DNA damage, indicating that DNA is one of the main targets for Cr(III) inside the cell (Plaper et al. 2002). Cr(VI) after being internalized in cells is reduced to Cr(III); RecG helicase might be involved in removing DNA damages induced by Cr(III), especially PALs that in vitro were proved to be DNA-ICLs. The data here obtained indicate that the RecG helicase has a crucial role in Cr(VI) resistance; Crg96 was the mutant with the highest Cr(VI)-sensitivity, and might have a pivotal role in solve PALs lesions induced by chromium.

Concluding, this study was made possible by two approaches: one was identification of the gene impaired in chromate-sensitive mutants obtained by transposon mutagenesis, and the other the examination of cellular phenotypes by a high-throughput technology defined PM. Results

obtained permitted us to correlate the functions of impaired genes to chromate resistance, and permitted to suppose that genes with different function are involved in chromate resistance in *P. corrugata* strain 28. *recG* helicase played a crucial role in counteracting chromate stress probably by solving PALs lesions due to Cr(VI)/Cr(III), and ME is probably involved in supplying NAD(P)H that is consumed by enzymes concerned in Cr(VI)-reduction; this function was strongly impaired in mutant and in biosynthetic pathways for rebuilding damaged cellular components.

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