Involvement of the *oscA* gene in the sulphur starvation response and in Cr(VI) resistance in *Pseudomonas corrugata* 28

Carlo Viti, Francesca Decorosi, Annalisa Mini, Enrico Tatti and Luciana Giovannetti

Correspondence
Carlo Viti
carlo.viti@unifi.it

Dipartimento di Biotecnologie Agrarie, Sez. Microbiologia, Università degli Studi di Firenze, Piazzale delle Cascine 24, 50144 Firenze, Italy

Pseudomonas corrugata 28 is a Cr(VI)-hyper-resistant bacterium. A Cr(VI)-sensitive mutant was obtained by insertional mutagenesis using EZ-Tn*5* <R6Kγori/KAN-2>Tnp. The mutant strain was impaired in a gene, here named *oscA* (<u>o</u>rgano<u>s</u>ulphur <u>c</u>ompounds), which encoded a hypothetical small protein of unknown function. The gene was located upstream of a gene cluster that encodes the components of the sulphate ABC transporter, and it formed a transcriptional unit with *sbp*, which encoded the periplasmic binding protein of the transporter. The *oscA*–*sbp* transcriptional unit was strongly and quickly overexpressed after chromate exposure, suggesting the involvement of *oscA* in chromate resistance, which was further confirmed by means of a complementation experiment. Phenotype MicroArray (PM) analysis made it possible to assay 1536 phenotypes and also indicated that the *oscA* gene was involved in the utilization of organosulphur compounds as a sole source of sulphur. This is believed to be the first evidence that *oscA* plays a role in activating a sulphur starvation response, which is required to cope with oxidative stress induced by chromate.

INTRODUCTION

Hexavalent chromium is a severe contaminant since, unlike trivalent chromium, it is highly water-soluble, toxic, mutagenic to most organisms, and carcinogenic for humans (Viti & Giovannetti, 2007). Because of its structural similarity to sulphate, chromate [Cr(VI)] crosses the cell membrane via the sulphate transport system (Cervantes *et al.*, 2001). Inside the cell, chromium toxicity is related to the process of the reduction of Cr(VI) to Cr(III). Cr(III) affects replication, causes mutagenesis, and alters the structure and activity of enzymes, reacting with their carboxyl and thiol groups (Cervantes *et al.*, 2001). Partial reduction of Cr(VI) generates Cr(V) and reactive oxygen species (ROS) (Cervantes *et al.*, 2001; Shi & Dalal, 1990a, b, 1992) associated with the establishment of oxidative stress that causes cellular damage of various kinds (De Flora, 2000; Petrilli & De Flora, 1977). The occurrence of Cr(VI) in the environment exerts a selective pressure on microbial communities (Viti & Giovannetti, 2001). Most micro-organisms are sensitive to Cr(VI), but some microbial groups are resistant and can tolerate high levels of chromate. In bacteria, Cr(VI) resistance is linked to plasmids and/or chromosomes. Various chromosomal resistance strategies have been reported, including: (i) modification of sulphate transport (Brown *et al.*, 2006; Hu *et al.*, 2005; Thompson *et al.*, 2007); (ii) extracellular reduction of Cr(VI) to Cr(III), which is trapped by anionic components of the bacterial cell envelope (Flemming *et al.*, 1990; McLean *et al.*, 1990; Snyder *et al.*, 1978); (iii) counteracting chromate-induced oxidative stress by activating enzymes involved in ROS scavenging (catalase, superoxide dismutase) (Ackerley *et al.*, 2006); (iv) the repair of DNA lesions by SOS response enzymes (RecA, RecG, RuvAB) (Hu *et al.*, 2005; Llagostera *et al.*, 1986; Miranda *et al.*, 2005); and (v) regulation of iron uptake, which may serve to sequester iron in order to prevent the generation of highly reactive hydroxyl radicals via the Fenton reaction (Brown *et al.*, 2006).

Although the antioxidant capabilities of reduced glutathione (GSH) have been clearly established, further study is needed to ascertain what protective role glutathione may play in counteracting chromate stress. In the cytoplasm, chromate probably interacts with GSH, leading to the formation of redox-cycling Cr(V) and consequently free radicals, an increased incidence of Cr(VI)-induced DNA strand breaks, and the formation of glutathione–Cr(III)–DNA adducts (Ackerley *et al.*, 2006). The capacity of GSH

to protect cellular constituents against oxidative stress is therefore in conflict with its direct interaction with chromium (Ackerley et al., 2006). Nevertheless, Helbig et al. (2008) found that the absence of GSH increased chromate toxicity, and hypothesized that the beneficial effect of GSH-mediated chromate detoxification might compensate for the production of free radicals (Helbig et al., 2008).

Plasmids may harbour genes that provide resistance to chromate by the efflux of the oxyanion from the cytoplasm (Nies, 2003; Pimentel et al., 2002).

Some studies have shown that chromate accumulates in cells by sulphate uptake systems, causing the upregulation of genes involved in sulphate transport and sulphur metabolism (Brown et al., 2006; Ramirez-Diaz et al., 2008). Moreover, chromate resistance in bacteria has been mainly examined in strains with a low chromate tolerance (Brown et al., 2006; Hu et al., 2005). In the present study, a Cr(VI)-sensitive mutant with impaired sulphur metabolism was obtained from a Cr(VI)-hyper-resistant (40 mM K_2CrO_4) isolate of Pseudomonas corrugata (Viti et al., 2006, 2007) using a transposon random insertion mutagenesis approach. The impaired gene and the flanking regions were sequenced and identified, and the mutant phenotype was compared with that of the wild-type strain using a high-throughput analysis called Phenotype MicroArray (PM). This approach revealed significant differences in sulphur metabolism between the mutant and the wild-type strain, and the role of the impaired gene having an unknown function was hypothesized. Transcriptional analysis of the impaired gene and the downstream genes was also carried out.

METHODS

Bacterial strains and plasmids. The strains of Pseudomonas corrugata and Escherichia coli and the plasmids used in this study are listed in Table 1.

Growth conditions and chromate minimal inhibitory concentration (MIC). P. corrugata strains were grown in LB (Sambrook et al., 1989) medium at 25 °C with shaking (200 r.p.m.). Tris-minimal medium (TMM) (Mergeay, 1995) plus 0.2 % (w/v) sodium gluconate and different concentrations of K_2CrO_4 (Merck) was used for the Cr(VI) susceptibility test. E. coli strains were grown in LB medium at 37 °C with shaking (200 r.p.m.).

Cr(VI) MIC was determined as described by Viti et al. (2003).

Chromate reduction. Cr(VI) reduction was investigated under aerobic conditions using the colorimetric diphenylcarbazide (DPC) method (Shen & Wang, 1995). Overnight cultures of the strains grown in TMM were diluted 1 : 50 in tubes containing TMM plus 0.2 mM K_2CrO_4. All cultures were incubated at 25 °C under shaking conditions (200 r.p.m.). A 1 ml sample from each culture was filtered (0.45 μm pore size) when the stationary growth phase was reached. The cell-free filtrate was subsequently mixed with 125 μl DPC reagent (Shen & Wang, 1995) and left at room temperature for 20 min. Cr(VI) was quantified by spectrophotometric measurements (UVICON spectrophotometer) at 540 nm. Growth medium interference was eliminated by using calibration curves obtained with medium without cells. Each determination was carried out on three replicates.

Competent cells of *P. corrugata* strain 28. To obtain electro-competent cells, strain 28 was grown in LB medium (100 ml) at 25 °C with shaking (200 r.p.m.) to the early exponential phase. Cells were harvested by centrifugation at 10 000 **g** for 10 min at 4 °C. The well-drained pellet was washed three times with 10 ml of 10 % (v/v) ice-cold glycerol. An 80 μl aliquot of ice-cold glycerol (10 %, v/v) was added to the pellet and then mixed. Competent cells were stored at −80 °C until used.

Transposon mutagenesis of *P. corrugata* strain 28 and selection of chromate-sensitive mutants. The EZ-Tn5 <R6Kγori/KAN-2>Tnp Transposome Mutagenesis kit (Epicentre Technologies) was used to generate Cr(VI)-sensitive mutants from strain 28. To obtain mutants, a 40 μl aliquot of electrocompetent cells was mixed with 20 ng transposome DNA (EZ-Tn5 <R6Kγori/KAN-2>Tnp Transposome Mutagenesis kit) and then electroporation was performed with a Gene Pulser Xcell electroporator (Bio-Rad) in 0.1 cm cuvettes at 2.0 kV, 125 μF and 200 Ω. Cells were resuspended in 2 ml SOC medium (Sambrook et al., 1989) and incubated at 30 °C for 1 h. Transformants were selected on TMM plates containing 15 μg

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Source or reference
Strains		
E. coli DH5α	Wild-type, F⁻ endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA–argF)U169 deoR φ80dlacΔ(lacZ)M15	New England Biolabs
E. coli DH5α($λ_{pir}$)	Strain to maintain R6Koriγ plasmid	Helinski et al. (1996)
P. corrugata 28	Wild-type, resistant up to 40 mM K_2CrO_4, isolated from a soil artificially polluted with 1000 mg Cr(VI) per kg soil	Viti et al. (2007)
Crg8 mutant	Derivative of P. corrugata strain 28 containing transposon Ez-Tn5, Km^r	This work
Plasmids		
pBBR1MCS-5	Broad-host-range cloning vector, Gm^r	Kovach et al. (1995)
pCHR61	oscA PCR fragment, including the putative promoter region, cloned into the ClaI site of pBBR1MCS-5	This work
pCHR62	oscA-sbp operon PCR fragment, including the putative promoter region cloned into the XhoI/BamHI sites of pBBR1MCS-5	This work

kanamycin ml^{-1} (TMM/Km). Colonies, which reached a diameter of 2–3 mm after 3-5 days incubation at 25 °C, were picked and plated onto TMM/Km plus 20 mM K$_2$CrO$_4$ and then incubated at 25 °C for 2 days. Clones unable to grow in the presence of 20 mM K$_2$CrO$_4$ were recovered and their Cr(VI)-susceptibility was reconfirmed.

DNA hybridization. To verify the single insertion of the EZ-Tn5 transposon in the genome of the selected mutant, DNA was isolated and digested with either *Pvu*II or *Mlu*I, which do not cut within the EZ-Tn5 transposon. DNA from strain 28 was used as a negative control. After electrophoretic separation of digests in a 0.8 % (w/v) agarose gel, Southern hybridization was performed as described by Sambrook *et al.* (1989). The probe used was a fragment of the kanamycin resistance gene of the EZ-Tn5 transposon amplified by using primers Kan-F and Kan-R (see Supplementary Table S1, available with the online version of this paper, for primer sequences) and then labelled with fluorescein by means of the Gene Images Random Prime Labelling kit (Amersham Life Sciences). Hybridization was performed at 55 °C; the blot was developed using the ECF detection system (Amersham Life Sciences) and subsequently scanned with a Typhoon 9200 (Amersham Life Sciences).

Identification of the transposon insertion site and sequencing of neighbouring regions. DNA was isolated from the mutant and digested with *Mlu*I, *Nhe*I or *Eco*RI, which do not cut within the EZ-Tn5 transposon. Restriction fragments were self-ligated using T4 DNA ligase. Plasmids were introduced into *E. coli* DH5α(λ_{pir}) by transformation of competent cells according to a standard procedure (Sambrook *et al.*, 1989). Transformants were recovered by plating on LB agar containing 50 μg kanamycin ml^{-1}. 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 kit. Subsequently, nucleotide sequences extending from the transposon insertion site were PCR-amplified using primers 8bfadf and cysAr (Table S1) designed on conserved regions found in *Pseudomonas* strains whose genome sequences have been published. The PCR products were purified by using a PCR Clean-up kit (Qiagen) and sequenced directly by primer walking.

Inverse PCR was used to isolate the sequence downstream of the cysAr primer annealing region from strain 28. An aliquot ($\approx$1 μg) of genomic DNA was digested in a 50 μl volume with *Cla*I. After heat-inactivation of the enzyme at 65 °C for 20 min, $\approx$100 ng DNA was diluted to a concentration of $\approx$2 ng μl^{-1} and ligated with 10 units of T4 DNA ligase (Roche Applied Science) at 16 °C overnight. The ligase mixture was concentrated to 25 μl by evaporation at 65 °C and then used as a template for a PCR using primers cysAf and cysAw1r (Table S1) in a 50 μl reaction volume. The PCR product was purified and sequenced by primer walking.

Sequencing was performed using a BigDye Terminator Cycle Sequencing kit on an ABI 3100 DNA sequencer (Applied Biosystems). The DNA sequences obtained were viewed and edited by using Chromas Lite (Technelysium) and aligned by using CLUSTAL_X (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/). ORFs were detected by using ORFFINDER (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The nucleotide sequence of each predicted ORF was analysed with the BLAST program in the GenBank database (National Center of Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov). Identification of operons was performed using the FGENESB pattern/Markov chain-based prediction program from Softberry (http://softberry.com/berry.phtml).

RNA extraction and cDNA synthesis. For RNA extraction 50 ml TMM medium (1.5 mM Na$_2$SO$_4$) in 250 ml side-arm Pyrex flasks (12 flasks in total) was inoculated with fresh overnight cultures (1 : 1000) of the wild-type and the mutant strains. The cultures were grown to

the early exponential phase (OD$_{600}$ 0.5), then K$_2$CrO$_4$ (final concentration 1 mM) was added to 10 flasks. The two cultures not exposed to chromate were immediately harvested and processed for RNA extraction, while the remaining five pairs were exposed to chromate for respectively 5, 15, 30, 60 and 90 min before harvesting and RNA extraction.

Total RNA was extracted with the RNeasy Bacterial kit (Qiagen) from 0.5 ml of each culture. Extracted RNAs were treated with DNase I (Roche) and subsequently quantified by agarose gel electrophoresis and a spectrophotometric reading (Biophotometer, Eppendorf). cDNA was prepared with 250 ng total RNA using 10 units of Superscript III (Invitrogen) and 10 μM random hexamers.

Transcriptional analysis. For verification of co-transcription of various ORFs, cDNA was amplified by PCR using primers designed to amplify the intergenic regions between *oscA* and *sbp*, *sbp* and *cysT*, *cysT* and *cysW*, *cysW* and *cysA* (Table S1). Reactions were carried out in the Thermal Cycler GeneAmp PCR System 9600 (Applied Biosystems) and consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C (the annealing temperature was reduced by 0.2 °C each cycle) for 30 s and extension at 72 °C for 60 s preceded by 5 min at 95 °C for initial denaturation and then by 7 min at 72 °C for final extension.

The expression level of *oscA*, *sbp* and *cysA* in cells exposed to chromate with respect to non-exposed cells was tested by real-time PCR. The Platinum SybrGreen qPCR Supermix UDG kit (Invitrogen) and iCycler (Bio-Rad) real-time PCR system were used. The primers used for the amplification of *oscA*, *sbp* and *cysA* are listed in Table S1. Real-time PCR was performed on three dilutions of each cDNA sample (1, 1 : 10, 1 : 100). The amplification conditions consisted of an initial 2 min denaturation step at 95 °C, followed by 45 cycles each of denaturation at 95 °C for 10 s, annealing at 56 °C for 10 s, and extension at 72 °C for 10 s. A negative control with PCR-grade water instead of cDNA template was included. At the end of the real-time PCR cycles, a melting curve was generated and analysed to confirm the product specificity. Raw data were analysed with Relative Expression Software Tool Multiple Condition Solver (REST-MCS) using the 16S RNA gene for normalization. For each sample, expression factor values were calculated from the results of three real-time PCR experiments performed on cDNA from RNA extracted from two independent cultures.

Genetic complementation. Plasmid pCHR61 was constructed by amplifying, with primers oscAf and oscAr (Table S1), a 1114 bp DNA fragment bearing a wild-type copy of the impaired gene. The fragment was subsequently digested with *Cla*I to release a 909 bp DNA fragment bearing the *oscA* gene and parts of the upstream and downstream ORFs (Fig. 1). The DNA fragment of 909 bp was cloned into the *Cla*I site of pBBR1MCS-5 and then the recombinant plasmids were screened for the orientation of the cloned DNA fragment with respect to the P*lac* promoter. Plasmid pCHR61, which harboured the DNA fragment in the same orientation as the P*lac* promoter, was used for complementation analysis.

Plasmid pCHR62 was constructed by amplifying an 1800 bp DNA fragment of strain 28 bearing the *oscA* and *sbp* genes and parts of the upstream and downstream ORFs (Fig. 1) with primers XhoIcysOPf and BamHIoscAspbr (Table S1). The fragment was then cloned in the *Xho*I/*Bam*HI sites of pBBR1MCS-5.

Plasmids pCHR61 and pCHR62 were transferred to *P. corrugata* strains by electroporation. Transformants were selected on LB medium amended with gentamicin (20 μg ml^{-1}).

Phenotype MicroArray-Biolog (PM) tests. The strains were tested on PM plates (PM01–PM04 and PM09–PM20) for 1536 different
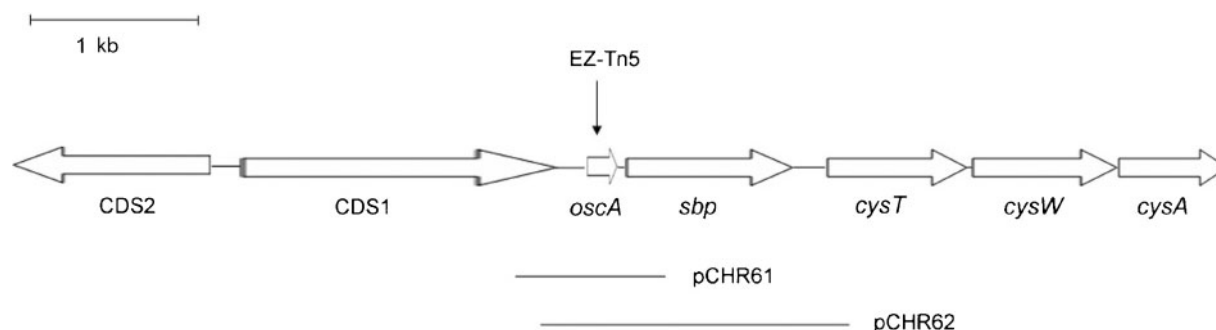
Fig. 1. Gene organization of the genome region sequenced in *P. corrugata* 28. *oscA*, encoding hypothetical small protein of unknown function; CDS1, encoding diguanylate cyclase/phosphodiesterase; CDS2, encoding fatty acid desaturase; *sbp*, encoding sulphate ABC transporter periplasmic sulphate-binding protein; *cysT*, encoding sulphate ABC transporter permease protein; *cysW*, encoding sulphate ABC transporter permease protein; *cysA*, encoding sulphate ABC transporter ATP-binding protein. The arrow indicates the position of transposon insertion. Solid black lines indicate the regions used for the construction of pCHR61 (harbouring the *oscA* gene) and pCHR62 (harbouring the *oscA* and the *sbp* genes).

conditions, including carbon, nitrogen, phosphorus and sulphur sources, several concentrations of ions and osmolytes, a wide variety of antibiotics, antimetabolites, heavy metals and other inhibitors, and pH stress. PM 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 colour that is recorded by a CCD camera every 15 min 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 analysed to compare the PM kinetics of different strains.

In order to perform PM experiments the wild-type strain and its mutant were grown overnight at 25 °C on BUG agar (Biolog) and then cells were picked up with a sterile cotton swab and suspended in 15 ml inoculation fluid (IF-0, Biolog). Cell density was adjusted to 85 % transmittance (*T*) on a Biolog turbidimeter. PM01–PM04 plates were inoculated (100 μl per well) with 85 % *T* cell suspensions mixed with 1 % (v/v) tetrazolium (DYEMIX A, Biolog). Inoculations for PM09–PM20 were carried out as described by Viti *et al.* (2007).

All the PM plates were incubated at 25 °C in an Omnilog Reader (Biolog) and monitored automatically every 15 min for colour changes in the wells. Readings were recorded for 48 h, and data were analysed with Omnilog-PM software (release OM_PM_109M) (Biolog), which generated a time-course curve for tetrazolium colour formation.

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

RESULTS

Isolation and characterization of a chromate-sensitive mutant

In order to learn more about the molecular mechanisms involved in chromate resistance in *P. corrugata* strain 28, for which the MIC of Cr(VI) was 40 mM, an attempt was made to select Cr(VI)-sensitive mutants after EZ-Tn5 mutagenesis. From a screening of over 14 000 transfor-

mants, eight clones with Cr(VI) MICs lower than 20 mM were found. After conducting a susceptibility test in liquid TMM, a stable mutant with a Cr(VI) MIC of 6 mM, hereafter called Crg8, was selected for further investigation.

An assay for Cr(VI) reduction, measured as a decline in the concentration of hexavalent chromium in the cultivation medium in which the initial concentration of Cr(VI) was 0.2 mM (as K_2CrO_4), showed that Cr(VI)-reducing activity was lower in the mutant than in the wild-type strain (32.7 ± 5.5 % and 52.3 ± 6.1 % respectively, with reference to the original chromate concentration in the medium).

Southern hybridization was performed using a portion of the kanamycin resistance gene of the EZ-Tn5 transposon as a probe. When the total DNA of Crg8 was digested with either *Pvu*II or *Mlu*I, which have no cut site in the EZ-Tn5 transposon, a single hybridization band was observed (data not shown), indicating that the transposon was integrated in the chromosome at a single site.

Identification of the impaired gene and flanking ORFs in the Crg8 mutant

To identify the gene affected by EZ-Tn5 insertion, the DNA regions flanking the transposon were sequenced (6950 bp) and the resulting data were compared with sequences available in the GenBank database.

Analysis of the sequence data led to the map of the predicted ORFs shown in Fig. 1. Transposon insertion had occurred in an ORF whose nucleotide sequence had 84 % identity with the sequence of a gene (PFL_0191) of *Pseudomonas fluorescens* Pf-5; hereafter this ORF will be called *oscA* (organosulphur compounds). The *oscA* ORF encodes a hypothetical small protein of unknown function, 60 amino acids in size and with a calculated molecular mass of 6.8 kDa. This hypothetical protein has no characterized domains and was classified by the Cluster

of Orthologous Group (COG) database as belonging to COG5583.

Upstream of *oscA* two ORFs were present, CDS1 and CDS2. CDS1 had the same orientation as *oscA,* and was similar to a diguanylate cyclase/phosphodiesterase with a PAS/PAC sensor(s)-encoding gene (80 % identity with *P. fluorescens* PfO-1, PflO1_0192). CDS2, which had an orientation opposite to that of *oscA,* showed a high similarity with a fatty acid desaturase-encoding gene (89 % identity with *P. fluorescens* PfO-1, PflO1_0191). The *oscA* ORF was followed by four ORFs which were similar to genes encoding the components of the sulphate ABC transporter: *sbp* (88 % identity with *P. fluorescens* PfO-1, PflO1_0194), *cysT* (89 % identity with *P. fluorescens* PfO-1, PflO1_0195), *cysW* (88 % identity with *P. fluorescens* Pf-5, PFL_0194) and *cysA* (89 % identity with *P. fluorescens* Pf-5, PFL_0195).

In order to gain insight into the function of the *oscA* gene, the presence of *oscA* homologues and the genetic arrangement downstream of *oscA* homologues in bacteria were investigated. Homologues of *oscA* were present in various Gram-negative and Gram-positive bacteria (see Supplementary Table S2), and the following downstream genes of *oscA* were found (Supplementary Fig. S1): (i) *sbp*, *cysT*, *cysW* and *cysA* genes, which encode the components of the sulphate ABC transporter in *Pseudomonas* strains, in *Shewanella oneidensis* MR-1, *Shewanella* sp. W3-18-1 and *Bacillus halodurans* C-125; (ii) genes involved in the transport and metabolism of sulphonate in *Methylobacillus flagellatus* KT, *Morella thermoacetica* ATCC 39073, and *Bacillus clausii* KSM-K16; (iii) genes encoding enzymes involved in the pathway of sulphate reduction that leads to cysteine in *Bacillus* strains. Only in the case of *Bacillus subtilis* subsp. *subtilis* 168 are there genes downstream of the *oscA* homologue apparently not involved in sulphur utilization.

Transcriptional analysis

The nucleotide sequence bearing the six identified ORFs was analysed by FGENESB operon prediction software. On the basis of the distances between the ORFs and the frequencies of the different genes neighbouring each other in known bacterial genomes, the software predicted the existence of two operons, one comprising *oscA* and *sbp*, and the other *cysT*, *cysW* and *cysA*. In order to confirm the operon prediction, RNA extracted from cultures of the wild-type strain non-exposed or exposed to chromate for different lengths of time (5, 30, 60 or 90 min) was retro-transcribed and then cDNA was used to amplify the intergenic regions between *oscA* and *sbp*, *sbp* and *cysT*, *cysT* and *cysW*, *cysW* and *cysA*. A PCR product was obtained for the intergenic regions *oscA–sbp*, *cysT–cysW* and *cysW–cysA*; no product was detected for the *sbp–cysT* intergenic region, or for the control samples prepared with non-retrotranscribed RNA (Fig. 2). These results indicated that *oscA* and *sbp* form an operon distinct from *cysTWA*.

Real-time RT-PCR experiments were carried out to evaluate the level of expression of *oscA*, *sbp* and *cysA* in the wild-type in response to Cr(VI). Table 2 shows the relative expression factors, calculated for the genes after 5, 15, 30, 60 or 90 min of Cr(VI) exposure and compared to the control condition [no Cr(VI) exposure]. All the genes were significantly overexpressed at three time points (5, 15 and 30 min), but the overexpression did not remain constant and decreased over time. The overexpression of the *oscA* gene was statistically significant up to the end of the experiment (90 min), though the level of *oscA* overexpression at this time was only 4 % of what it was after 5 min of Cr(VI) exposure. The *sbp* and *cysA* genes in the mutant strain were significantly overexpressed after only 5 min of chromate exposure (Table 2).
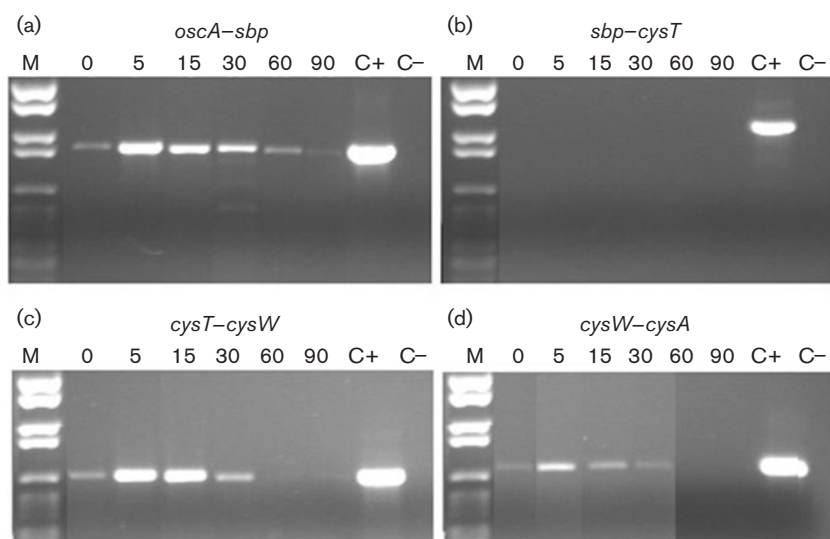

Fig. 2. RT-PCR of the intergenic regions *oscA–sbp* (a), *sbp–cysT* (b), *cysT–cysW* (c) and *cysW–cysA* (d). For each intergenic region, primers were designed inside the coding sequence of the two neighbouring genes. RNA was isolated from cultures of the wild-type strain exposed to chromate (1 mM) for 15, 30, 60 or 90 min. C+, positive control (genomic DNA of *P. corrugata* 28); C−, negative control (non-retrotranscribed RNA); M, DNA molecular mass marker VI (Roche).

Table 2. Expression of *oscA*, *sbp* and *cysA* after different durations of Cr(VI) exposure in the wild-type and in the Crg8 mutant strains

Strain	Gene	Expression factor from real-time RT-PCR*				
		5 min	15 min	30 min	60 min	90 min
Wild-type strain 28	*oscA*	115.9	41.6	27.7	9.0	4.7
	sbp	135.4	44.5	3.7	NS	NS
	cysA	32.1	19.6	7.8	NS	NS
Crg8 mutant	*sbp*	8.5	NS	NS	ND	ND
	cysA	9.1	NS	NS	ND	ND

*Relative expression factor in chromate-exposed cultures compared with non-exposed cultures as calculated by MCS-REST software. All numerical values are statistically significant ($P=0.01$). For each sample, expression factor values were calculated from the results of three real-time PCR experiments performed on total RNA extracted from two independent cultures. NS, Not significantly different ($P>0.05$); ND, not determined.

PM characterization

The wild-type and its mutant were phenotypically characterized with the innovative PM high-throughput technique. The metabolic panels PM01–PM04 and PM09–PM10 and the sensitivity panels PM11–PM20 were used to monitor 1536 phenotype characters (detailed information about the PM panels is available at http://www.biolog.com).

By analysing the consensus data (obtained from two independent experiments), the phenotypes gained or lost by the mutant as compared with the wild-type were identified (Table 3).

The largest number of phenotypes lost by the mutant as compared with the wild-type was related to sulphur compound utilization (PM04 wells F1–H12). Compared to the wild-type, Crg8 maintained its capacity to use inorganic sulphur compounds, cysteine and some of its derivatives (L-cysteinyl-glycine, L-cysteine sulphinic acid), lanthionine and djenkolic acid, but it did not utilize *S*-methyl-L-cysteine, cystathionine, glutathione, methionine and all its tested derivatives, taurine, hypotaurine, butanesulphonic acid, 2-hydroxyethanesulphonic acid or methanesulphonic acid.

The comparison of the kinetic curves obtained on PM01 and PM02 plates, which tested the capacity of cells to utilize different carbon sources, showed that the mutant had a lower activity on D-saccharic acid than the wild-type, and a higher activity on L-arabinose and D-galacturonic acid.

The results of the PM03 plate, which tested the capacity of cells to use different nitrogen compounds as a sole nitrogen source, showed that the mutant was impaired in its utilization of ammonia and acetamide. No differences were found between the wild-type and the mutant as regards phosphorus compound utilization (PM04 wells A1–E12), or by the PM09 and PM10 plates, assaying osmolytes and the pH gradient, respectively.

The sensitivity panels (PM11–PM20) enabled 960 different tests to be carried out. As expected, the mutant, which harbours the kanamycin resistance gene of the EZ-Tn5 transposon, had gained resistance to kanamycin and other aminoglycoside antibiotics (data not shown). The amino-hexose moiety of these antibiotics contains a 3'-hydroxyl group which is a substrate of the 3'-aminoglycoside phosphotransferase encoded by the kanamycin resistance gene located in the Ez-Tn5 transposon used for the mutagenesis experiment. Resistance to these aminoglycoside antibiotics gained by the mutant is therefore likely to be due to acquisition of the kanamycin resistance gene. The mutant was more sensitive than the wild-type to two quinolone antibacterials (norfloxacin and pipemidic acid), which inhibit DNA topoisomerases, and to the fungicides dodine and captan, which are cytotoxic.

Genetic complementation

Complementation experiments were performed to verify the involvement of *oscA* in the two major phenotypes lost by the mutant as compared with the wild-type: the Cr(VI) hyper-resistance and the capacity to use various organo-sulphur compounds as a sole source of sulphur. The plasmids pBBR1MCS-5 (vector plasmid), pCHR61 (harbouring the *oscA* gene) and pCHR62 (harbouring the *oscA* and *sbp* genes) were transferred into the mutant and the wild-type. pBBR1MCS-5 had no effect on the phenotype of the mutant or the wild-type strains. Plasmids pCHR61 and pCHR62 allowed a partial recovery of Cr(VI) resistance in the mutant, but caused a decrease in the Cr(VI)-resistance level of the wild-type. It is possible that the introduction of pCHR61 and pCHR62, leading to inappropriate copy numbers of *oscA* or *oscA–sbp* genes respectively, induces an imbalance of their functions.

The Cr(VI)-reduction test showed that pBBR1MCS-5 did not have any effect on the Cr(VI) reduction (data not shown). The insertion of pCHR61 and pCHR62 into the mutant and the wild-type caused more Cr(VI) to disappear from the growth medium: the presence of pCHR61 and pCHR62 in the wild-type increased the Cr(VI) reduction

Table 3. Results of PM comparison of the *P. corrugata* Crg8 mutant with its wild-type parent strain 28

Test	Substrate/chemical	Mode of action	Phenotype of Crg8*
Metabolism (PM01–PM04 and PM09–PM10)			
Phenotypes gained – Faster Growth/Resistance	L-Arabinose	Carbon source	+94
	D-Galacturonic acid	Carbon source	+69
Phenotypes lost – Slower Growth/Sensitivity	D-Saccharic acid	Carbon source	−101
	Ammonia	Nitrogen source	−102
	Acetamide	Nitrogen source	−123
	S-Methyl-L-cysteine	Sulphur source	−164
	Cystathionine	Sulphur source	−185
	Glutathione	Sulphur source	−125
	L-Methionine	Sulphur source	−128
	D-Methionine	Sulphur source	−152
	Glycyl-L-methionine	Sulphur source	−107
	N-Acetyl-DL-methionine	Sulphur source	−158
	L-Methionine sulphoxide	Sulphur source	−150
	Taurine	Sulphur source	−131
	Butanesulphonic acid	Sulphur source	−115
	2-Hydroxyethanesulphonic acid	Sulphur source	−123
	Methanesulphonic acid	Sulphur source	−141
Chemical sensitivity (PM11–PM20)			
Phenotypes lost – Slower Growth/Sensitivity	Norfloxacin	DNA topoisomerase, quinolone	−283
	Pipemidic acid	DNA unwinding, gyrase (GN), topoisomerase (GP), quinolone	−227
	Dodine	Fungicide, guanidine, membrane permeability	−188
	Captan	Fungicide, carbamate, multisite	−171

*The OmniLog-PM software generates time-course curves for respiration (tetrazolium colour formation) and calculates differences in the areas for the mutant and the wild-type strains. The units are arbitrary. Positive values indicate that the mutant showed higher rates of respiration than the wild-type strain; negative values indicate that the wild-type strain showed higher rates of respiration than the mutant. The differences are averages of values reported for two replicates.

from $52.3 \pm 6.1\%$ to $75.2 \pm 1.0\%$ and $66.8 \pm 1.8\%$ (with reference to the original chromate concentration in the medium, 0.2 mM K_2CrO_4), respectively, whereas in the mutant the Cr(VI) reduction increased from $32.7 \pm 5.5\%$ to $37.5 \pm 1.7\%$ and $52.5 \pm 4.3\%$, respectively. The greater amount of Cr(VI) that disappeared from the growth medium of the strains complemented with pCHR61 and pCHR62 as compared with the non-complemented wild-type and mutant strains could be a consequence of increased Cr(VI) uptake followed by intracellular reduction.

The mutant transformed with pCHR61 and analysed by PM (PM04, sulphur compound utilization) showed a complete recovery of all lost phenotypes, yielding the same profile as the wild-type strain (Fig. 3).

DISCUSSION

Sequencing of the DNA regions flanking the transposon insertion in the Crg8 mutant [obtained from the Cr(VI) hyper-resistant bacterium *P. corrugata* 28] indicated that the impaired gene, here called *oscA*, encoded a hypothetical small protein of unknown function. Data obtained by PM (1536 phenotypic tests) and genetic approaches made it possible to explore the function of the impaired gene, and provided new insight into the processes involved in chromate stress and the mechanisms for counteracting it.

Complete sequencing of *oscA* and flanking genes showed that *oscA* was located upstream of a gene cluster encoding the components of the sulphate ABC transporter, where: (i) *sbp* encodes the periplasmic sulphate-binding protein, (ii) *cysT* and *cysW* encode the two inner-membrane transport proteins, and (iii) *cysA* encodes the membrane-associated ATP-binding protein.

Co-transcriptional analysis of the *oscA*, *sbp*, *cysT*, *cysW* and *cysA* genes showed that *oscA* forms a transcriptional unit with *sbp*, while *cysT*, *cysW* and *cysA* constitute a second transcriptional unit.

The expression patterns of the above-mentioned genes in cultures grown with and without chromate were evaluated by real-time RT-PCR. The presence of chromate induced a fast and high overexpression of the *oscA* and *sbp* genes in
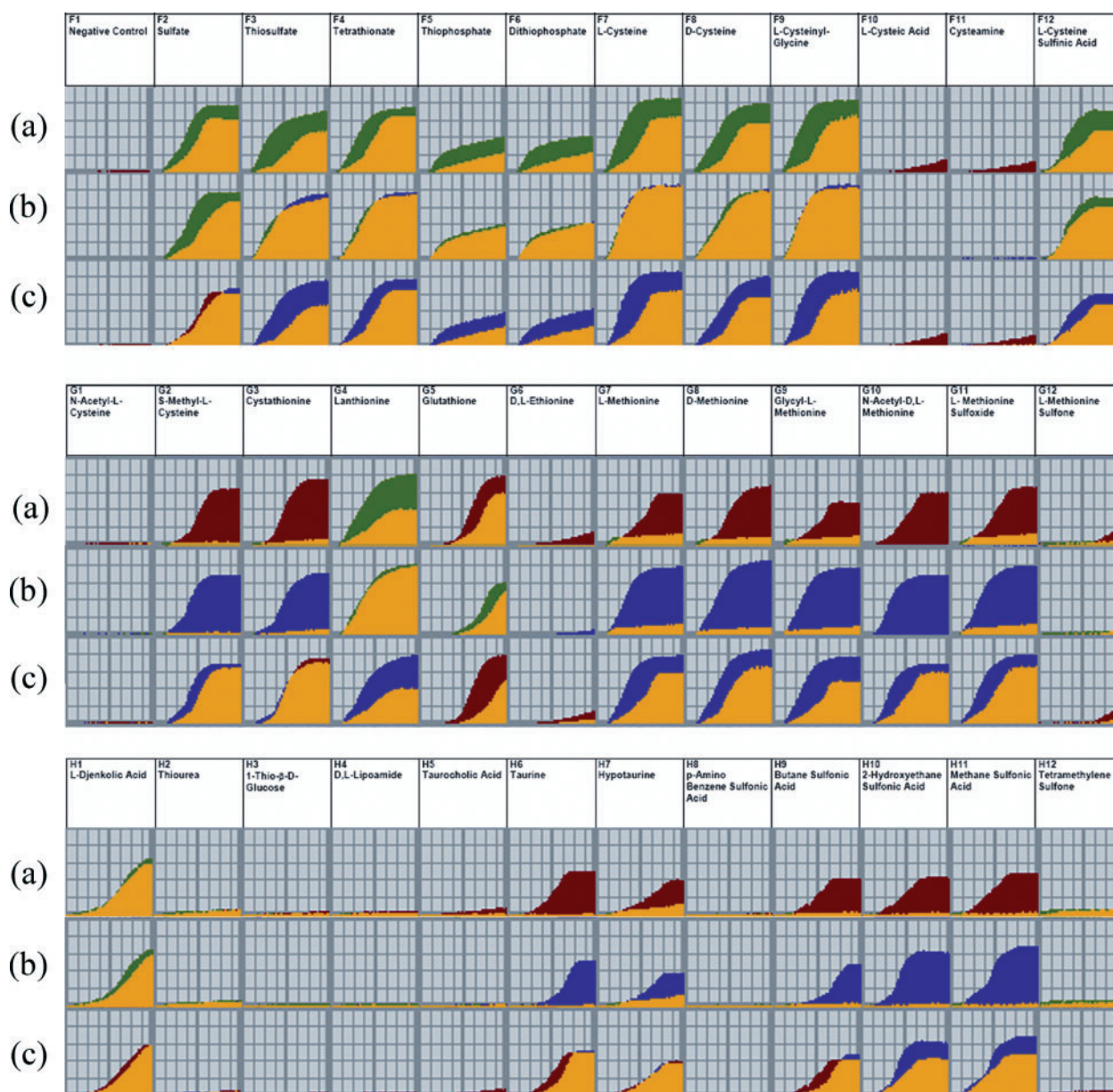
Fig. 3. PM kinetics for sulphur compound utilization (PM04) showing the consensus data for a comparison of the wild-type strain versus the Crg8 mutant (a), the Crg8 mutant versus the Crg8 mutant complemented with pCHR61 harbouring the *oscA* gene (b), and the wild-type strain versus the Crg8 mutant complemented with pCHR61 (c). For each well the sulphur compound tested is indicated. OmniLog-PM software attributes red, green and blue colours to the kinetic curves of the wild-type strain, the Crg8 mutant and the Crg8 mutant complemented with pCHR61, respectively. The overlapping curves of the two strains are indicated in orange.

strain 28, suggesting that *oscA* could be a key gene in chromate resistance. This hypothesis is supported by the transcriptome analysis carried out on *Shewanella oneidensis* MR-1, which showed the upregulation of an *oscA* homologue (SO4651) during the early response to chromate exposure (Brown *et al.*, 2006).

The expression pattern of *cysA* in strain 28 after chromate addition highlighted that the *cysTWA* operon was over-

expressed with a temporal expression pattern that resembled that observed for *oscA–sbp*, but with a lower level than that found for the *oscA–sbp* transcript. This provided a strong indication that strain 28 responded to chromate by modulating sulphate uptake. When the external chromate concentration is high, competition for sulphate transport takes place between the sulphate and the chromate. Since the amount of sulphate that crosses the membrane is inadequate for the sulphate assimilation

pathway, a sulphate starvation condition occurs. In this condition, the upregulation of *cysTWA*, which encodes the sulphate transporter core, leads to an increase in the membrane of the sulphate ABC transporters that are made especially selective for sulphate by the overexpression of *sbp*. For although SBP binds both chromate and sulphate, it has a higher affinity for sulphate (Jacobson *et al.*, 1991). The greater expression of *sbp* and *cysTWA* in chromate-exposed strain 28 could be an attempt to cope with the sulphur starvation induced by the chromate. An increase in sulphate transport has been extensively reported in sulphur-starved bacterial cells (Cuhel *et al.*, 1981; Green & Grossman, 1988; Jeanjean & Broda, 1977). The sulphur-starvation condition is also induced by oxidative stress due to chromate exposure (Brown *et al.*, 2006; Mostertz *et al.*, 2004; Pinto *et al.*, 2004; Salunkhe *et al.*, 2005). There are a variety of possible explanations for this: (i) disruption of intracellular redox cycling, leading to insufficient sulphite reduction; (ii) leakage of sulphide following cell envelope damage (Benov *et al.*, 1996); (iii) increased demand for protective compounds containing thiols (Fahey 2001); and (iv) increased biosynthesis of Fe–S clusters that are sensitive to disruption by reactive oxygen (Layer *et al.*, 2007).

In the presence of chromate the Crg8 mutant showed a reduction in *sbp* overexpression compared with the wild-type strain. This suggested that the impairment of *oscA* had a polar effect on *sbp* transcription and could explain the chromate sensitivity of the mutant, since *sbp* plays a crucial role in chromate/sulphate uptake modulation, as mentioned above. In the Crg8 mutant the transcription of the *cysTWA* operon after chromate exposition also decreased compared with the wild-type, suggesting that the products of *oscA* and/or *sbp* were required for the physiological overexpression of *cysTWA* in response to chromate. We hypothesize that the regulation of *cysTWA* expression in response to chromate depends on *oscA*, because the role of the sulphate-binding protein is to bind with high affinity the sulphate ions in periplasmic space. In a study on *S. oneidensis* MR-1, the genes linked to sulphate transport and sulphur metabolism were found to be upregulated in the presence of chromate (Brown *et al.*, 2006). In addition to evidence of the overexpression of genes involved in sulphur metabolism, for the first time we identified the organization of *oscA*, *sbp*, *cysT*, *cysW* and *cysA* in two operons (*oscA–sbp* and *cysTWA*). There was also a strong indication that the former regulated the expression of the latter in the presence of chromate.

The PM technique produced a detailed and comprehensive phenotypic profile of strain 28 and its mutant, and provided insight into the function of *oscA* in sulphur utilization and the response to chromate. The mutant strain showed a very defective spectrum of sulphur compound utilization as a sole source of sulphur, lacking the ability to use several organosulphur compounds. When Crg8 was complemented with pCHR61, bearing the *oscA* gene, on the other hand, it fully recovered its ability to use

these substrates as a sole source of sulphur. Therefore our study shows that *oscA*, a gene of unknown function, plays an essential role not only in sulphate uptake when there is sulphur limitation, but also in the utilization of organo-sulphur compounds in *P. corrugata* strain 28. Since methionine, alkanesulphonates, glutathione and taurine are used by bacterial cells as a response to sulphur starvation conditions (Hummerjohann *et al.*, 1998; Kahnert *et al.*, 2000; van der Ploeg *et al.*, 1996), and since the PM indicated that the utilization of these organosulphur substrates depended on *oscA*, this gene could be a key gene for the sulphur starvation response. It is still unclear how the pseudomonads recognize sulphur starvation, and how they subsequently control the expression of a variety of organosulphur-assimilating pathways (Endoh *et al.*, 2005; Kertesz, 2000; Tralau *et al.*, 2007). For instance, in *Pseudomonas aeruginosa* the pathway of sulphur compound utilization in the absence of sulphate is complex and involves the upregulation of 132 genes, including a homologue of *oscA* (PA0284) (Tralau *et al.*, 2007). To our knowledge, this is the first study to report that the *oscA* gene functions are indispensable for organosulphur compound assimilation.

The differences between the mutant and the wild-type strains found by PM in the utilization of three carbon sources and two nitrogen sources might be due to the carbon, the nitrogen or the sulphur metabolism links. However, a good deal of work will be required to establish any role for *oscA* in carbon and nitrogen metabolism.

Chemical sensitivity characterization of the mutant obtained by the PM experiments (PM09–PM20) provided evidence for the belief that the role of *oscA* in chromate resistance is due to its role in countering the effects of sulphur starvation arising from Cr(VI)-induced oxidative stress. Compared with strain 28, the mutant showed an increased sensitivity to norfloxacin, pipemidic acid and captan, which induce oxidative stress (Dwyer *et al.*, 2007; Suzuki *et al.*, 2004), and consequently sulphur starvation in the cells. The mutant was also more sensitive to dodine, which severely damages cytoplasmic membranes, causing them to lose their selective permeability and leading to the leakage of several metabolites (Brown & Sister 1960; Cabral, 1991) that may induce sulphur starvation in the cells (Benov *et al.*, 1996).

In conclusion, the mutagenesis data, the transcriptional analysis and the phenomic approach showed that *oscA* is a key gene for chromate resistance in strain 28. *oscA* is located upstream of a gene cluster that encodes the components of the sulphate ABC transporter, and it forms a single transcriptional unit with *sbp*, which encodes the periplasmic sulphate-binding protein. It has been shown that *oscA* is essential for the utilization of various organosulphur compounds and that it induces upregulation of the *cysTWA* operon under conditions of chromate exposure. *oscA* may also have a regulatory role in the sulphur starvation response, but the exact mechanism

through which it acts is not well understood and deserves further study. The capacity of *oscA* to induce chromate resistance may be due to its role in remedying the effects of sulphur starvation arising from chromate exposure.

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