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Genome analysis and characterization of zinc efflux systems of a highly zinc-resistant bacterium, *Comamonas testosteroni* S44

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Abstract

A novel and multiple metal(loid)-resistant strain *Comamonas testosteroni* S44 with a high Zn^{2+} resistance level (10 mM) was isolated. To understand the molecular basis for the high zinc resistance, whole genome sequencing was performed and revealed a large number of genes encoding putative metal(loid) resistance proteins, mobile genetic elements (MGEs) and horizontal gene transfer (HGT) events that may have occurred to adapt to a metal(loid)-contaminated environment. In particular, 9 putative Zn^{2+} transporters [4 *znt* operons encoding putative Zn^{2+}-translocating P-type ATPases and 5 *czc* operons encoding putative RND-driven (resistance, nodulation, cell division protein family)] tripartite protein complexes were identified. Real-time RT-PCR analysis revealed that the four *zntA*-like genes were all induced by Zn^{2+}, while *czcA* genes were either Zn^{2+}-induced or downregulated by Zn^{2+}. Furthermore, a *zntR*1*A*1 operon encoding a ZntR-type regulator and a P-type ATPase was studied in detail. The *zntR*1 deletion strain (S44Δ*zntR*1) displayed intermediate resistance to Zn^{2+} (6 mM) and accumulated more intracellular Zn^{2+}. Reporter gene expression assays indicated that ZntR1 responded to Zn^{2+}, Cd^{2+} and Pb^{2+}, with Zn^{2+} being the best inducer. Gene transcription analysis indicated that ZntR1 was a regulator for transcription of *zntA*1, while other putative ZntR-type regulators may also regulate the transcription expression of *zntA*1.

Keywords: Zinc resistance; *znt* operon; *czc* operon; Whole genomic sequence

1. Introduction

In all organisms, zinc acts as an important trace element which participates in a large number of metabolic reactions. Zinc functions as coenzyme, cofactor or stabilizing protein structure (Vallee and Auld, 1993). Microorganisms need to take up zinc as a micronutrient for vital cell functions. However, zinc is toxic in excess of normal physiological levels, since it interacts with sulfhydryl groups or replaces other essential metals in a wide range of proteins (Fosmire, 1990; Kox et al., 2000). Therefore, microbial regulatory systems are necessary to maintain intracellular zinc levels.

Bacteria have the ability to tightly regulate zinc homeostasis using import and export systems. After reaching a threshold concentration of zinc entering the cell, expression of the uptake transporters may be repressed and the efflux transporters induced. In *Escherichia coli*, the *znuABC* operon encoding the high affinity Zn^{2+} uptake system is negatively controlled by Zur (Patzer and Hantke, 1998), acting as a repressor to prevent entry of RNA polymerase into promoter regulating *znuABC* (Shin et al., 2007). Zn^{2+} export systems include P-type ATPases, RND-transport systems and CDF transporters (Hantke, 2001). Genes encoding Zn^{2+} translocating P-type ATPases include

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zntA, *pbrA*, *cadA* and *czcP* (Nies, 2003; von Rozycki and Nies, 2009), which are responsible for exporting Zn^{2+} efficiently, even extracting metal ions bound to thiol groups (Rensing et al., 1999). These genes encoding divalent metal-transporting P-type ATPases are usually regulated by ZntR, a MerR-type family member that acts as a repressor but is converted into a transcriptional activator in the presence of Zn^{2+}/Cd^{2+}/Pb^{2+} (Outten et al., 1999). In some bacteria, Zn^{2+}, Pb^{2+} and Cd^{2+} are also extruded by the same ZntA-like protein, but the main function of ZntA-like proteins appears to be Zn^{2+} transport (Legatzki et al., 2003a). Proteins encoded by *czc* operons constitute a branch of metal-transporting RND-type efflux systems which typically encode a metal-specific multi-component efflux pump CzcCBA. Often, *czcCBA* operons are regulated by an adjacent two-component regulatory system, CzcRS (Legatzki et al., 2003a). Sometimes, there are additional genes such as *czcN* and *czcI* with their promoters *czcNp* and *czcIp* that are located upstream of CzcCBA (Groβe et al., 1999). In *Cupriavidus metallidurans* CH34, transcription of the operon depends on regulation of CzcR, which binds to the *czcNp* promoter region, providing a regulatory path CzcS→CzcR→*czcNp* (Groβe et al., 2004). Some metal-resistant bacteria harbor multiple efflux systems and models of their interplay have been studied. For example, *C. metallidurans* CH34 contains 4 *zntA*-like genes that may have been duplicated during evolution of the strain, and at least 3 operons encoding Czc-type RND systems are located on the chromosome and two on the megaplasmid pMOL30. One chromosomal *czc* operon is induced by Zn^{2+} but only to a moderate degree, one is constitutively expressed, while the third *czc* operon is silent. However, the two *czc* operons located on pMOL30 are vigorously expressed when cells are treated with Zn^{2+} (von Rozycki and Nies, 2009). Though the Czc system is able to export Zn^{2+} efficiently from the periplasm (Legatzki et al., 2003a), ZntA was suggested to be the preliminary line of defense before the CzcCBA efflux system can be effective (Nies, 2003), since ZntA transports Zn^{2+} from the cytoplasm into the periplasm (Rensing et al., 1999).

Though there have been many studies on bacterial zinc homeostasis, the molecular details of regulation and interplay of those different transporters in response to Zn^{2+}, Pb^{2+}, Cd^{2+} or other metals have not been well characterized thus far, especially in the *Comamonas* genus. Recently, the genomes of two *Comamonas* species were sequenced. One is *Comamonas testosteroni* KF-1 (GenBank number, AAUJ02000001), for which 3 ZntA-like genes and 1 CzcCBA-like operon were predicted (Schleheck et al., 2004). In another strain, *C. testosteroni* CNB-2 (CP001220), eight putative ZntA-like genes and 6 CzcCBA operons were found (Ma et al., 2009). However, this was solely based on in silico predictions since their physiological function has not been investigated.

The objective of this study was to identify important components responsible for bacterial zinc resistance and gene regulation of a highly zinc-resistant *Comamonas* strain. Our results provide insight into adaptive processes such as a mobile gene pool that is likely in constant rearrangement in response to an environment contaminated with multiple metal(loid)s.

2. Materials and methods

2.1. Bacterial isolation and identification

Heavy-metal-contaminated mining soil with mining waste was collected from an antimony mine in Lengshuijiang City, central south China (27°46′N and 111°28′E), where the soil was severely contaminated with multi-metal(loid)s. The average concentrations of Zn, Sb, Cd, Pb and As were 149.08, 1825.47, 13.08, 71.27 and 82.64 mg/kg, respectively (She et al., 2010). After Zn^{2+} enrichment, several zinc-resistant bacteria were isolated onto chemically defined medium (CDM) plates with 5 mM $ZnCl_2$ (Weeger et al., 1999). The minimal inhibitory concentrations (MICs) were determined (Lim and Cooksey, 1993) with serial concentrations of Zn^{2+}, Cd^2, Ni^{2+}, Co^{2+}, Cu^{2+}, As^{3+}, Sb^{3+} and Hg^{2+} in liquid CDM or Tris minimal medium for Pb^{2+} (Mergeay et al., 1985). One strain, named S44, showed multiple resistances to metal(loid)s, especially Zn^{2+} and was chosen for further study.

The colony morphologies of strain S44 were observed on LB plates. Biochemical and physiological characteristics were determined using the API 20NE system (bioMérieux, Marcy l'Etoile, France). The partial 16S rRNA gene was first generated by PCR and later the full sequence was generated using the whole genome sequence.

Bacteria and plasmids used in this study are listed in Table 1. Strains S44 and S44Δ*zntR*1 were grown at 28 °C in LB with or without Zn^{2+}. *E. coli* DH5α containing different plasmids was grown at 37 °C in LB medium. Antibiotics were used at the following final concentrations: chloramphenicol, 50 μg/ml, ampicillin, 100 μg/ml and kanamycin, 100 μg/ml.

2.2. Whole genomic sequencing analysis

High-molecular-mass genomic DNA isolated from strain S44 using a blood and cell culture DNA mini-kit (Qiagen, Maryland, USA) was used to construct a 4–40 kb random genomic library. Whole genome shotgun sequencing was performed by the University of Arizona Genetics Core Facility using a Roche 454 Genome Sequencer FLX instrument. The

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Description	Reference or source
C. testosteroni S44	Wild type, Zn^{2+} resistance, Chl^s	This study
C. testosteroni S44Δ*zntR*1	*zntR*1 deletion, Chl^r	This study
E. coli DH5α	TA cloning or expression host	Promega
pGEM-T	Amp^r, PCR TA cloning vector	Promega
pMod-OCm	Chl^r, template to amplify *chl* gene	Dennies and Zylstra, 1998
pCM-184	Amp^r, Kan^r, Tet^r, cloning vector	Marx and Lidstrom, 2002
pGL4.17[luc2/Neo]	Amp^r, promoterless reporter vector	Promega
pGL-zntR1	Amp^r, PzntR1 report vector	This study

Amp^r, Chl^r, Kan^r, Tet^r = ampicillin, chloramphenicol, kanamycin and tetracycline resistance, respectively.

assembled contigs were submitted to the RAST annotation server for subsystem classification and functional annotation (Aziz et al., 2008). Sequence alignments and co-linearity comparison were performed by GenomComp 1.3 (Yang et al., 2003) and ACT release 9.0 (Carver et al., 2005). Sequence similarities were searched using BlastN and BlastP (http://www.ncbi.nlm.nih.gov/BLAST) and alignments were performed using the ClustalW algorithm (Thompson et al., 1994). GC content was analyzed using the CLC Main Workbench 5 program (http://www.clcbio.com). The NCBI Prokaryotic Genomes Automatic Annotation Pipeline was used for gene annotation in preparation for data submission to GenBank (http://www.ncbi.nlm.nih.gov/genomes/static/Pipeline.html).

2.3. Construction of a zntR1 deletion in strain S44

Construction of a *zntR*1 disruption plasmid from strain S44 was performed as reported previously (Kothe et al., 2004). Briefly, (1) a 1.7 kb fragment including *zntR*1 (420 bp) and its flanking DNA sequence (642 bp upstream of the TGA stop codon and 636 bp downstream of the GTG start codon) was amplified by PCR and cloned into pGEM-T; (2) a defined 221-bp deletion was created in the *zntR* coding sequence by inverse PCR with primers kzntF and kzntR, generating a 4.5 kb product that was then digested with XbaI; (3) a promoterless 750 bp Chl^r-resistant gene fragment was amplified from pMod-Ocm (Table 1) and cloned into the 4.5 kb fragment to generate pGEM-*zntR*1; (4) pGEM-*zntR*1 was digested with MunI and SacI and cloned into the same restriction-enzyme-treated pCM184 to generate suicide plasmid pCM-*zntR*1 (Marx and Lidstrom, 2002). The *zntR*1 disruption plasmid was transferred into strain S44 by electroporation with 1.8 kV for 5.2 ms (GenePulser™ Bio-rad), and the resulting double recombination mutant strain of the *zntR*1 locus was named S44Δ*zntR*1(Fig. S1, Supplementary materials). MICs of S44Δ*zntR*1 to different metals were determined as described above.

2.4. Transcriptional expression analysis of genes encoding ZntA- and CzcA-like proteins

The PCR primers used in this study are listed in Table S1. The culture of strain S44 was grown to early exponential phase in LB medium, then amended with Zn^{2+} to a final concentration of 2 mM and further incubated for 20 min. Total RNA was prepared using the TRIzol method following the manufacturer's instruction (Invitrogen, Carlsbad, USA). Specific primers (Table S1) were designed and used in real-time RT-PCR for quantitative analysis of the expression levels. The reaction was performed using the SYBR green real-time PCR master mix (Toyobo, Osaka, Japan) using a BioRad option real-time PCR machine (BioRad, Berkeley, CA, USA). 16S rRNA genes (16S rDNA) were amplified as controls. The results were analyzed using MJ Option Monitor™ Analysis software 3.1 (BioRad). For transcription comparison of *zntR*1 between strain S44 and strain S44Δ*zntR*1, the two cultures were grown as described above and treated with 0.5 mM EDTA or 2 mM Zn^{2+}.

2.5. Detection of the response of ZntR1 to Zn^{2+} and other metals

To determine whether the regulatory protein ZntR1 responds to Zn^{2+} or other metals, a luciferase gene reporter assay was designed in such a way as to simulate the transcription model of *zntR*1*A*1 (Fig. 2A) using *luxAB* to substitute for *zntA*1. The *zntA*1 and *zntR*1 transcript were made in an opposite direction and each had its own putative promoter. A 560 bp fragment containing the overlapped *zntA*1 and *zntR*1promoters and the *zntR*1 in strain S44 was amplified, then digested with *NheI* and *HindIII* and cloned into promoterless reporter vector pGL4.17 [luc2/Neo] (Promega, Madison, WI USA) previously treated with the same restriction enzymes and transferred to *E. coli* DH5α by electroporation. The resulting plasmid was named pGL-*zntR*1 in which the *zntA*1 promoter was located upstream of the luciferase gene *luxAB*. After the strain reached early exponential phase with $OD_{600} = 0.1$ in LB, 1 mM metal ions (Zn^{2+}, Cu^{2+}, Cd^{2+}, Ni^{2+} or Pb^{2+}) or 0.5 mM EDTA were added, respectively. The cultures were collected, washed and treated with Dual-Glo™ Luciferase assay system (Promega), the activity of LuxAB (relative light units, RLUs) was measured using a Modulus™ luminometer (Promega) at 28 °C within 2 h.

2.6. Measurement of Zn^{2+} concentration in bacterial cells

Strains S44 and S44Δ*zntR*1 were each grown to exponential phase, then amended with $ZnCl_2$ to a final concentration of 1 mM and incubated for another 5 h. The cells of each culture were harvested and washed twice with 20 mM HEPES-NaOH (pH 8.0) containing 10 mM EDTA to remove externally bound metal ions. The cells were broken using a sonicator (Branson, Sonifier 450, Danbury, CN, USA) and centrifuged at 15,000 g for 10 min. The liquid phase was filtrated with a 0.22 μm filtration membrane (Millipore, Shanghai, China). Zn^{2+} content in the cells was determined using an atomic absorption spectrophotometer (Pgeneral, TAS-986, Beijing, China).

2.7. Deposition of nucleotide sequences

C. testosteroni S44 was deposited in the ARS Culture Collection (NRRL, http://nrrl.ncaur.usda.gov/) with collection number NRRL B-59472. The whole shotgun genome sequence of S44 has been deposited at DDBJ/EMBL/GenBank under accession ADVQ00000000. The version described in this paper is the first version, ADVQ0100000.

3. Results

3.1. A highly zinc-resistant strain, C. testosteroni S44 was isolated

Strain S44 was isolated from metal(loid)-contaminated soil with high zinc resistance. The colonies on LB agar plates were white, circular, smooth and convex. In liquid CDM medium

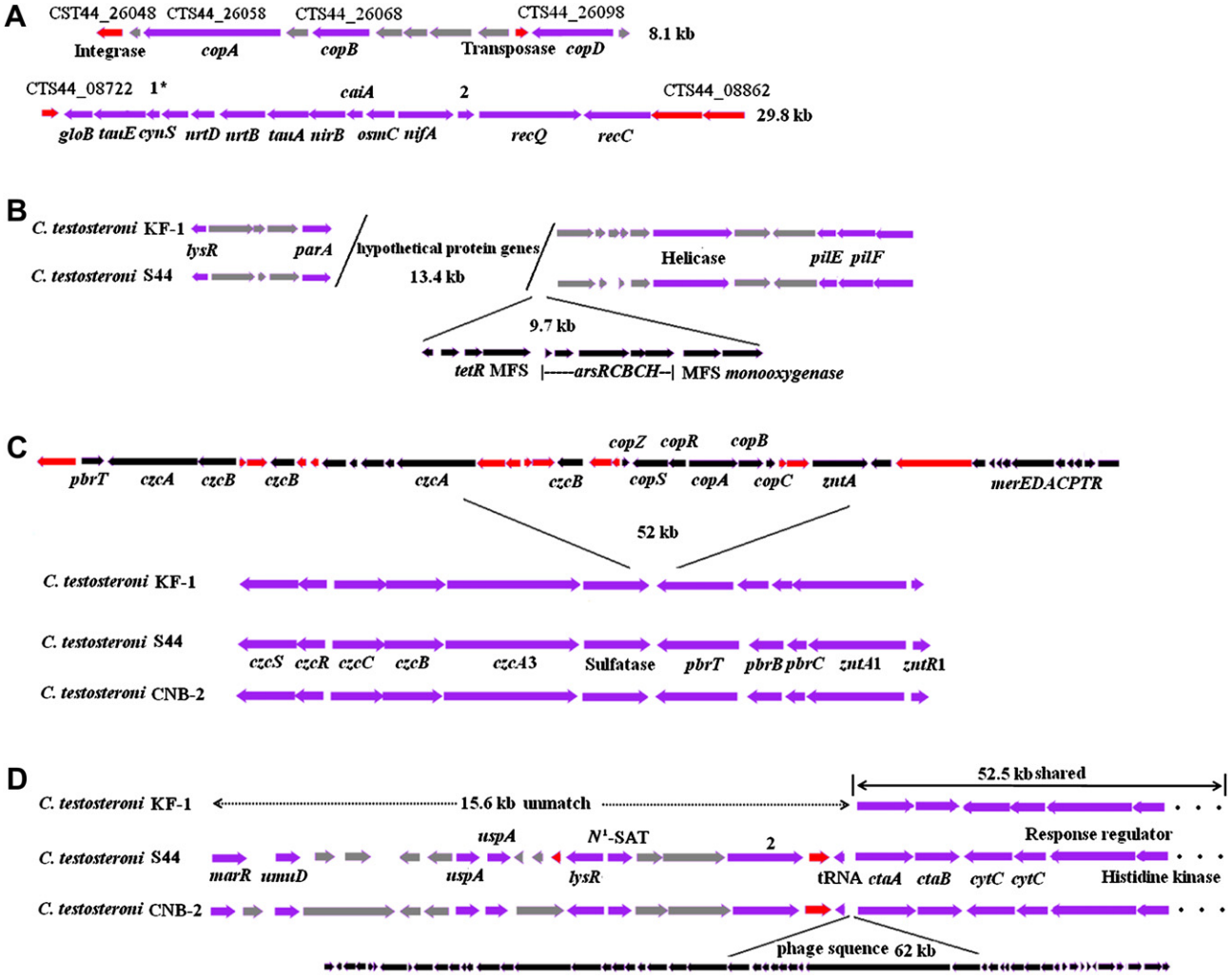

Fig. 1. Comparison of putative genetic determinants of mobile, heavy metal resistances and prophage sequences among *C. testosteroni* strains S44 (this study), KF-1 and CNB-2. (A) Two putative HGT gene clusters with flanking genes encoding transposases in strain S44. (B) A unique putative HGT arsenic-resistant operon in strain S44. (C) A big insertion between metal-resistant determinants in strain KF-1 with multiple mobile genetic elements. (D) Repeated *uspA* (universal stress protein) identified in strains S44 and CNB-2. The gray, purple, red and black background boxes represent hypothetical protein genes, known function genes, mobile genetic elements and unique genes observed within single strains, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Tris minimal medium for Pb^{2+}), the MICs of strain S44 for Zn^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+}, As^{3+}, Sb^{3+}, Hg^{2+} were 10.0, 2.0, 3.5, 3.0, 3.0, 4.0, 20.0, 0.3 and 0.1 mM, respectively. 16S rRNA gene analysis showed that the most closely related species of S44 was *C. testosteroni* strain KS0043 (M11224) with a 100% nucleotide similarity.

Physiological and biochemical analyses (API 20NE test) showed that strain S44 was oxidase-positive, capable of nitrate reduction and assimilation of adipate, caprate, gluconate and malate, but negative for indole production, acidification of glucose, arginine dihydrolase, citrate, urease, β-glucosidase, proteaseand assimilation of glucose, maltose, mannose, mannitol phenyl-acetate and N-acetyl-glucosamine. These results of physiological and biochemical analyses of strain S44 were in agreement with the typical characteristics of *C. testosteroni* (Garrity et al., 2002). Based on the above morphological, physiological/biochemical characteristics and 16S rRNA gene sequence, this strain was identified and renamed *C. testosteroni* S44.

3.2. Genome analysis of C. testosteroni *S44 indicates recent HGT*

The whole genomic sequence of *C. testosteroni* S44 contained 5,534,155 bp and an average GC content of 61.42%, which is similar to the two published *C. testosteroni* members, CNB-2 (CP001220) (Ma et al., 2009) and KF-1 (AAUJ02000001) (Schleheck et al., 2004). *C. testosteroni* S44 contained 5218 predicted open reading frames (ORFs) and 64 tRNA genes for all 20 amino acids and 5 scattered rRNA genes (Table S2). The likely origin of replication (*oriC*) was located between *rpmH* and *dnaA*, co-localization with downstream *dnaN* and *gryB* genes. Remarkably, large numbers of mobile genetic elements (MGEs), including prophages, various transposases and integrases, were predicted in *C. testosteroni* S44 (Table S3). Physiological analysis showed that *C. testosteroni* S44 had poor capacity to assimilate carbohydrates (only adipate, caprate and gluconate), which was in accordance with *C. testosteroni* CNB-2 (Ma et al.,

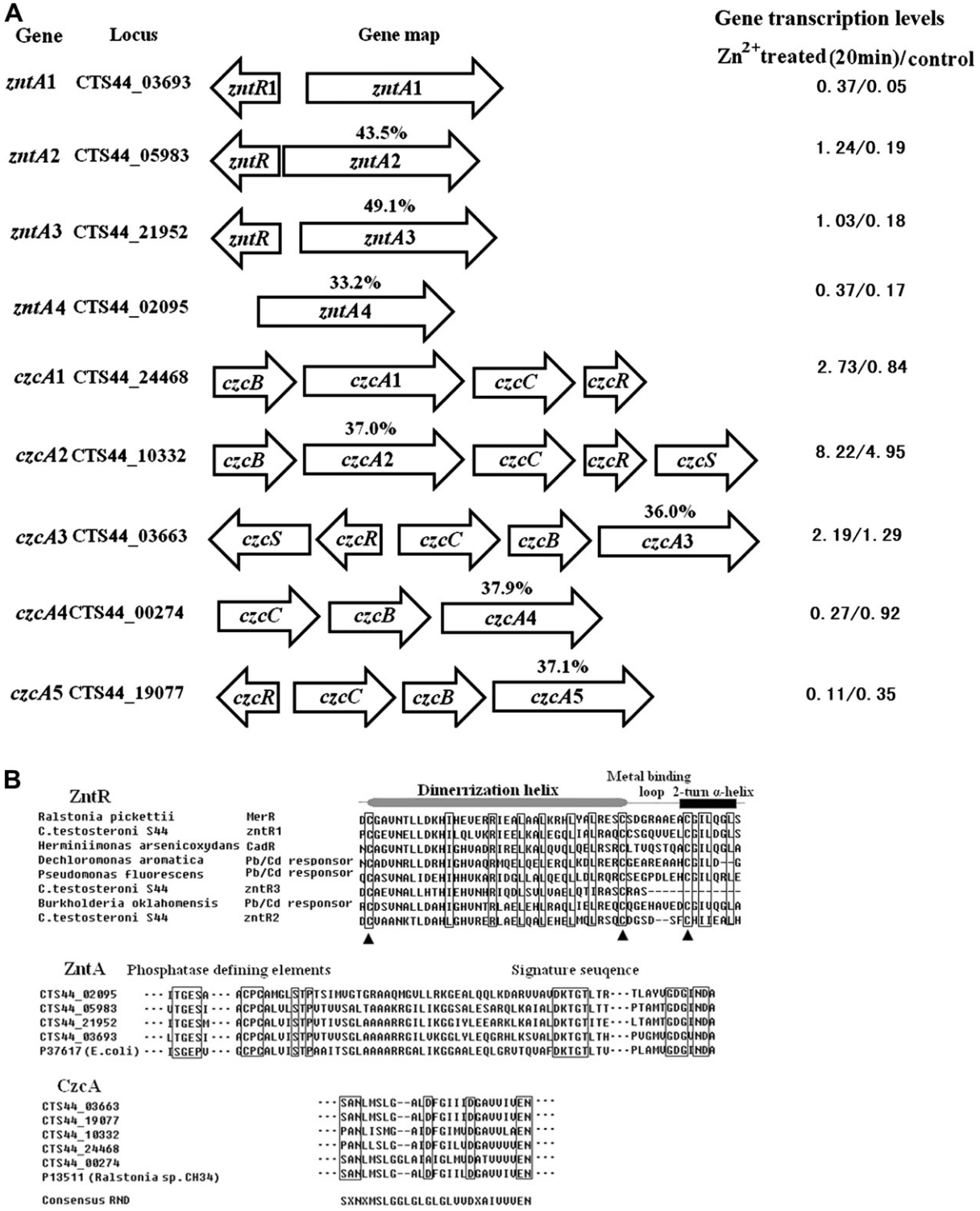

Fig. 2. Genome analysis of putative Zn^{2+} efflux operons of strain S44. (A) Physical maps of the 9 putative Zn^{2+} efflux operons (left) and their Zn^{2+}-treated transcription patterns (on the right is real-time RT-PCR analysis of transcriptional levels (the average of triplicate samples) of Zn^{2+} induced strains and non-induced strains for each ZntA or CzcA gene. The estimation of relative abundance of mRNA was normalized with the 16S rRNA genes and calculated using the ΔCt method (Pfaffl, 2001). The percentages above *zntA*/*czcA* are the amino acid identities compared with ZntA1 and CzcA1, respectively. (B) Conserved functional amino acid residues of the putative ZntR, ZntA and CzcA proteins in strain S44 and other strains.

2009). Gene operons encoding enzymes responsible for aromatic compound degradation, such as testosterone, benzoate, protocatechuate and gentisate, were detected in the *C. testosteroni* S44 genome that were also observed in *C. testosteroni* KF-1 and CNB-2. However, a unique hydrolase gene was only observed in the protocatechuate degradation operon of *C. testosteroni* S44, which might promote the metabolism of protocatechuate (Ma et al., 2009). Phenol degradation operons were found in *C.*

testosteroni strains S44 and CNB-2, but were absent in strain KF-1 (Fig. S2).

More importantly, a broad range of genes encoding determinants related to metal(loid) resistances with highest similarity to gene products from *C. testosteroni* KF-1 or CNB-2, including 4 ZntA-like P-type ATPases and 5 CzcA-like RND-transporters that are most likely involved in Zn^{2+} efflux, could be identified (Table S4). A putative copper resistant operon (clusters CTS44_26048 to CTS44_26098, 8.1 kb) and determinants with multiple functional genes (including ion transport, nitrite metabolism and DNA helicase genes, clusters CTS44_08722 to CTS44_08862, 29.8 kb) were unique in *C. testosteroni* S44 and were not detected in strains KF-1 and CNB-2 (Fig. 1A), indicating a very recent HGT event, since genes encoding integrase and transposase were located on both termini with a lower GC (57.1% and 46.7%) content compared to the whole genome (61.4%). In addition, a 9.7 kb insertion containing a putative arsenic resistance operon was detected in *C. testosteroni* S44 that had large shared flanking sequences with strain KF-1 or CNB-2 (Fig. 1B). Furthermore, some unique operons were identified in strain KF-1 or CNB-2, e.g. a big insertion (52 kb) that may confer resistances for Zn^{2+}, Cu^{2+} and Hg^{2+} in strain KF-1 (Fig. 1C), a phage insertion (62 kb) in strain CNB-2 (Fig. 1D). Strain S44 and CNB-2 shared repeated *uspA* genes encoding a universal stress protein, while strain KF-1 was missing such a fragment (Fig. 1D). Interestingly, the three β-proteobacteria *Delftia acidovorans* SPH1, *C. metallidurans* CH34 and *C. testosteroni* KF-1 shared two genomic islands (DAGI-1 and DAGI-2) with a putative function in metal resistance (Van Houdt et al., 2009; Janssen et al., 2010), while those determinants were not found in *C. testosteroni* S44 and CNB-2.

3.3. Not all genes encoding predicted Zn^{2+} efflux operons were induced by Zn^{2+}

C. testosteroni S44 contained 4 *zntA* genes encoding putative Zn^{2+} translocating P-type ATPases and 5 *czcA* genes encoding the central RND-type $Co^{2+}/Zn^{2+}/Cd^{2+}$ efflux pump (Fig. 2A). The physical maps of the *zntA* operons were regular, as expected with three of the four *zntA* genes having the putative regulator gene *zntR* adjacent to *zntA* (Fig. 2A, *zntA*1-3). In contrast, gene arrangements for the 5 *czc* operons were variable (Fig. 2A). The encoded functional amino acid residues of ZntR, ZntA and CzcA proteins were detected in all selected examples (Fig. 2B). However, the 4 ZntA proteins displayed relatively low amino acid similarities to each other, as did the CzcA proteins (Fig. 2A), which enabled us to design gene-specific primers to evaluate their transcriptional induction. In addition, one *czc* operon (*czcA*3) was located close to a *zntA* operon (*zntA*1). This arrangement was highly conserved among the three strains except for the insertion in strain KF-1 (Fig. 1C). A similar arrangement could be detected in the metal-resistant strain *C. metallidurans* CH34 with a *czcCBA*-like operon downstream of a corresponding, reversely oriented operon encoding a P-type ATPase (von Rozycki and Nies, 2009; Janssen et al., 2010).

To understand the molecular basis for zinc resistance, we studied gene expression using gene-specific primers in real-time RT-RCR. All 4 genes encoding ZntA-like proteins showed Zn^{2+}-induced expression, even *zntA*4, which was without an adjacent gene encoding a regulator (Fig. 2A). In contrast, the transcription patterns of the different *czcA* genes were variable; *czcA*1, *czcA*2 and *czcA*3 were Zn^{2+}-induced, while *czcA*4 and *czcA*5 were downregulated by Zn^{2+} (Fig. 2A).

In operon *zntR*1*A*1, each gene contained a putative promoter (partially overlapping), which was divergently transcribed. ZntR1 of *C. testosteroni* S44 encoded a 139 aa protein that shared 97% identity with a putative transcriptional regulator of the MerR family from *C. testosteroni* CNB-2 (ACY33209), and 34% identity with the Hg^{2+} resistance regulator MerR from *E. coli* (Lund and Brown, 1989). ClustalW algorithm revealed that ZntR was related to some MerR or $Zn^{2+}/Cd^{2+}/Pb^{2+}$ regulators, especially in functionally conserved aa residues (Fig. 2B). The other two putative ZntR-type regulators (ZntR2 and ZntR3) also contained similar conserved aa sequences with ZntR1 (Fig. 2B).

3.4. Disruption of zntR1 made C. testosteroni S44 more sensitive to Zn^{2+}

The *zntR*1 gene encoding a putative regulator was partially deleted by the homologous shuttle suicide plasmid pCM-*zntR*1 that contained a 750 bp Chl^r cassette. After electroporation, double recombinants were obtained and the *zntR* deletion was confirmed by the phenotype of Chl^r resistance and PCR analysis. The PCR amplification fragment of the *zntR*1 deletion strain (S44Δ*zntR*1) was 750 bp longer than that of S44, indicating insertion of the Chl^r sequence (Fig. 3A). Such insertion was further improved by sequencing analysis (data not shown). The MIC values for Zn^{2+}, Pb^{2+} and Cd^{2+} of *C. testosteroni* S44Δ*zntR*1 were lower than those of *C. testosteroni* S44 (10 mM vs 6.0 mM, 2 mM vs 1.5 mM and 3.5 mM vs 2.0 mM, respectively), while the MICs of Ni^{2+} and Cu^{2+} in S44Δ*zntR*1 were not decreased.

3.5. ZntR regulates Zn^{2+} detoxification

cDNA of *zntR*1 was detected in *C. testosteroni* S44 but was absent in *C. testosteroni* S44Δ*zntR*1 (data not shown), further confirming the deletion of *zntR*1. Both *zntA*1 (Fig. 2A) and *zntR*1 (Fig. 3B) were strongly induced by Zn^{2+}, indicating they were associated with Zn^{2+} detoxification. When excess Zn^{2+} (2 mM) was present, *zntA*1 showed increased transcriptions in both *C. testosteroni* S44 (1.71 times) and *C. testosteroni* S44Δ*zntR*1 (1.93 times, Fig. 3B), indicating other ZntR-like regulators might also regulate the transcription of *zntA*1. In addition, when cells were treated with EDTA, the transcription level of *zntA*1 was decreased in *C. testosteroni* S44, but slightly higher in S44Δ*zntR*1 (Fig. 3B).

3.6. ZntR1 was responsive to Zn^{2+}, Pb^{2+} and Cd^{2+}

To determine whether regulatory protein ZntR1 responds to Zn^{2+} or other metals, we constructed luciferase reporter

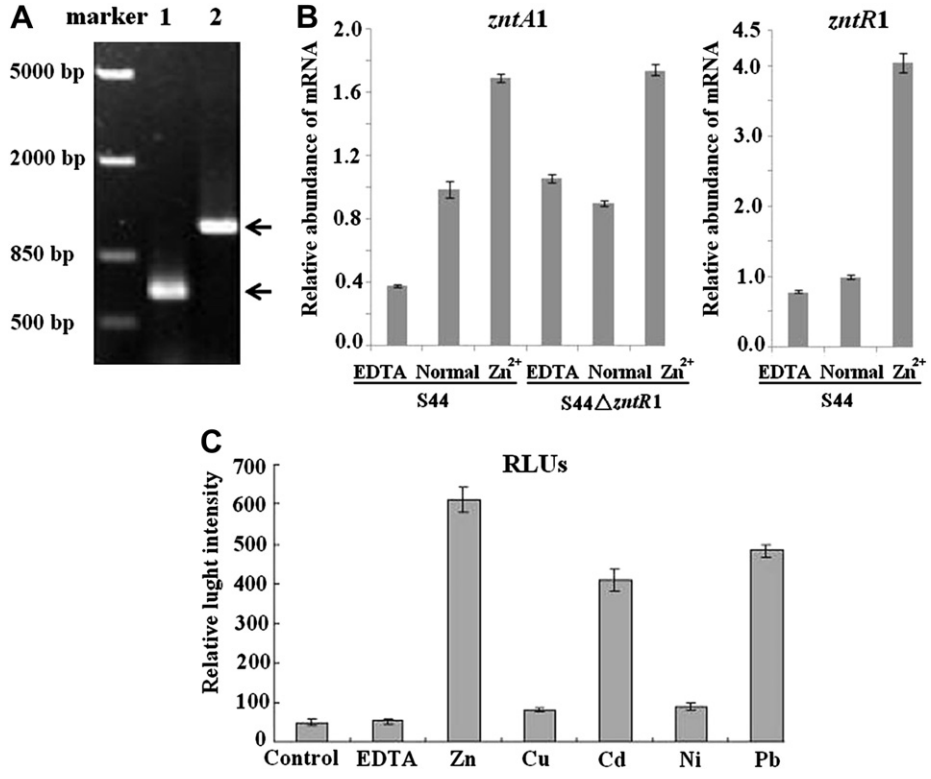

Fig. 3. Detection of *zntR*1 deletion and response to heavy metals. (A) PCR products obtained from *C. testosteroni* S44 (lane 1) and S44Δ*zntR*1 DNA (lane 2). The PCR-amplified fragment (≈1350 bp) of S44Δ*zntR*1 (lane 2) was 750 bp (Chl ^{r} cassette insertion) longer than that of strain S44 (≈600 bp). (B) The real-time RT-PCR transcription levels of *zntA*1 and *zntR*1. The *Y* axis represents the relative abundance of mRNA of *zntA*1 or *zntR*1 in treatments (2 mM Zn^{2+} or 0.5 mM EDTA) and in the control (normal LB). The estimation of relative abundance of mRNA was carried out as described above. The error bars indicate standard deviations of triplicate PCR samples. (C) Luciferase activity (reflected by relative light units) assay by reporter plasmid pGL-*zntR*1 in *E. coli* DH5α. The control represents the *E. coli* strain containing pGL-*zntR*1 without metal treatments.

plasmid pGL-zntR1. The RLUs, which reflect firefly luciferase activity, were determined. When cells were treated with zinc, RLUs increased about 11-fold compared to the control (Fig. 3C). When the chelator EDTA was added, RLUs were slightly lower than the control, showing a background level of expression due to trace amounts of zinc in the medium.

We also tested whether ZntR1 responded to other divalent metal ions such as Cu^{2+}, Cd^{2+}, Ni^{2+} and Pb^{2+}. As shown in Fig. 3C, RLUs increased about 7- and 9-fold when adding 1 mM Cd^{2+} or Pb^{2+}, respectively. However, there were no significant RLUs changes when adding 1 mM Cu^{2+} or Ni^{2+}. Hence, ZntR1-dependent induction was not only responded to by Zn^{2+}, but also by Cd^{2+} and Pb^{2+}, while Zn^{2+} may the optimal inducer.

3.7. The intracellular concentration of Zn^{2+} in strain S44ΔzntR1 was higher than in strain S44

To further confirm the importance of zinc transport regulated by ZntR1, the zinc content in *C. testosteroni* S44 and *C. testosteroni* S44Δ*zntR*1 cells was determined. After 5 h incubation with 1 mM Zn^{2+}, the Zn^{2+} concentration of *C. testosteroni* S44Δ*zntR*1 and of *C. testosteroni* S44 was 0.333 μM/OD cells (SD = 0.001, triplicate samples) and 0.244 μM/OD cells (SD = 0.031), respectively. The Zn^{2+} concentration in *C. testosteroni* S44Δ*zntR*1 was slightly higher (1.36 fold) than that in *C. testosteroni* S44, confirming ZntR1-dependent activation of *zntA* expression, which contributed to high Zn^{2+} resistance. However, since there are other putative Zn efflux systems, the deletion of *zntR*1 did not dramatically increase the intracellular Zn concentration.

4. Discussion

In this study, the bacterium *C. testosteroni* S44 with very high Zn^{2+} resistance level was isolated. Furthermore, this strain also harbored multi-metal(loid) resistances, especially for Cd^{2+}, Pb^{2+}, Cu^{2+} and As^{3+}. Most previous reports regarding various strains of *Comanonas* were related to degradation of complex aromatic compounds or their application in remediation of contaminated soil (Liu et al., 2007; Ma et al., 2009), while multiple metal resistance capacity was poorly studied. However, many contaminated sites contain both heavy metals and organic waste, so it is of interest to understand both resistance to metals and the ability to degrade aromatic compounds for improved outcome in remediation. *C. testosteroni* S44 was isolated from heavily multiple-metal(loid)-contaminated soils. It was therefore reasonable to speculate that the multiple high metal(loid) resistances of strain S44 could have been shaped by long-term selection and adaptation to a metal(loid)-contaminated environment, since the isolated site has been mined for more than 110 years (She et al., 2010). HGT might bring about distinct

changes in the GC content of the genome and provide additional physiological capacity to the receiving cells, such as heterologous insertion of the arsenic-resistant operon that may be responsible for high As^{3+} resistance (20 mM), the *osmC* gene with a putative peroxiredoxin function that was shown to be responsible for removal of reactive oxygen species (ROS) in *E. coli* (Shin et al., 2004). Heavy metal stress could induce the production of ROS and disrupt cellular redox status in algae (Pinto et al., 2003); thus, genes acquired by HGT encoding proteins that help cope with stress caused by ROS might mitigate the damage of metal stress and enable strain *C. testosteroni* S44 to survive in a metal(loid)-contaminated environment. Most metal resistance genes in strain S44 shared highest similarities with *C. testosteroni* KF-1 or CNB-2, but not with other genera. Those genes might have originated from a common ancestor during evolution of the genus *Comamonas*.

Transcriptional patterns of the different *zntA* and *czcA* genes were quite different, indicating a varied response of *C. testosteroni* S44 to Zn^{2+} detoxification. All 9 genes were transcribed without adding Zn^{2+} to LB. The 4 putative *zntA* genes were induced by addition of 2 mM Zn^{2+}, including *zntA*4, which was not found to have an adjacent regulator. This indicated that other ZntR-like proteins may also regulate transcription of *zntA*4. A previous study showed that Zn^{2+} was able to promote conversion of apo-ZntR to zinc-bound ZntR (Cui et al., 2008), which strongly activated transcription of *zntA* to expel Zn^{2+} from cells (Outten et al., 1999).

In the case of *czcA* genes, three *czcA* genes showed induction, while the other two were downregulated by Zn^{2+}. Such results were similar to a previous study which examined metal-resistant *C. metallidurans* CH34, in which one *czc* operon was induced by Zn^{2+} but only to a moderate degree, one was constitutively expressed, and the third *czc* operon was silent (von Rozycki and Nies, 2009). It has been reported that when several highly related genes were present, one was usually induced by heavy metals, while the other one or two were silent or constitutively expressed (Nies et al., 2006). *C. testosteroni* S44 may require constitutive expression of *czcCBARS* as a defense in a metal-contaminated environment, and expression of the other *czc* operons may be needed for detoxification of Zn^{2+} or other metals, such as Cd^{2+} and Pb^{2+}. The high number of such transporters indicates that they may play diverse roles in metal ion homeostasis (Gaither and Eide, 2001). ZntA may also be necessary to pump out ions bound to thiolate complexes, especially when Zn^{2+} or Cd^{2+} are present at a concentration of less than 200 μM, which would be below the response concentration of the *czcCBA* system (Legatzki et al., 2003b). Furthermore, ZntA was reported to be a highly specific efflux system for Zn, since it is only active when sufficient amounts of Zn^{2+} are bound to its N-terminal histidine residues and at least one ZntA was required to reach full Cd^{2+} resistance in *Ralstonia metallidurans* (Legatzki et al., 2003a). Thus, it might be critical that all *zntA* genes be tightly regulated; thus, transcription of the 3 *zntA* genes with regulators was only strongly induced when treated with 2 mM Zn^{2+}. The members of the cation diffusion facilitator (CDF) family that might be involved in zinc homeostasis could also be identified in the genome of strain S44 (Table S4). However, P-type ATPases like ZntA are much stronger transporters and are thus more important in resistance to high concentrations of metals such as zinc. CDF transporters are more important in zinc homeostasis at or near physiological levels.

In *C. testosteroni* S44Δ*zntR*1, *zntA*1 was still induced by Zn^{2+}, probably due to the presence of other regulators, which confirmed by genomic analysis that there are two additional genes encoding putative ZntR regulators. In addition, *zntA*1 itself had a putative promoter, which may be induced by Zn^{2+}. Furthermore, the *zntA*1 expression level decreased when treated with the metal-chelating agent EDTA, but not in S44Δ*zntR*1, which provided evidence that transcription of *zntA*1 and *zntR*1 was a response to Zn^{2+} or other metals. The mutant was more sensitive to Zn^{2+} and accumulated more intracellular Zn^{2+} due to reduced expression of *zntA* genes. Real-time RT-PCR results showed that when there was deficient Zn^{2+} (treated with EDTA), ZntR1 showed relatively weak expression. Upon addition of Zn^{2+}, ZntR1 may undergo a conformational change, thereby pulling the unusually spaced promoter together and subsequently enabling RNA polymerase to bind and vigorously express ZntA1 to export Zn^{2+} out of the cells (Outten et al., 1999; Singh et al., 1999).

Here we successfully identified important components responsible for Zn^{2+} resistance and gained insight into Zn^{2+} regulation within a novel highly zinc-resistant *Comamonas* species. The large number of genes encoding putative divalent metal efflux proteins and their variable expression patterns, coupled with a high number of other metal(loid)-resistance-related genes in the whole genome sequence, suggested sequential adaptation of *C. testosteroni* S44 to survive in metal-contaminated environments. Perhaps the different transport systems have different affinities for ionic Zn^{2+} or various components bound to zinc. This, in turn, might allow better fine-tuning, and subsequently, better ability to tolerate higher concentrations of various metal(loid)s.

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Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.resmic.2011.06.002.

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