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ORIGINAL PAPER

A heavy-metal tolerant novel bacterium, *Alcaligenes pakistanensis* sp. nov., isolated from industrial effluent in Pakistan

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Abstract Two strains, NCCP-650^T and NCCP-667, were isolated from industrial effluent and their taxonomic positions were investigated using a polyphasic taxonomic approach. The strains were found to be Gram-stain negative, strictly aerobic, motile short rods, which are tolerant to heavy-metals (Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}). Cells were observed to grow at a temperature range of 10–37 °C (optimal 25–33 °C), pH range of 5.5–10.0 (optimal 6.5–7.5) and can tolerate 0–7 % NaCl (w/v) (optimum 0–1 %) in tryptic soya agar medium. Sequencing of the 16S rRNA gene and two housekeeping genes, *gyrB* and *nirK*, of the isolated strains revealed that both strains belong to the *Betaproteobacteria* showing highest sequence similarities with members of the genus *Alcaligenes*. The chemotaxonomic data [major quinones as Q-8; predominant cellular fatty acids as summed features 3 ($C_{16:1}$ ω7c/iso-$C_{15:0}$ 2OH) and $C_{16:0}$ followed by Summed features 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3OH), $C_{17:0}$ Cyclo and $C_{18:1}$ ω7*c*; major polar lipids as diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and one unidentified aminolipid] also supported the affiliation of the isolated strains with the genus *Alcaligenes*. DNA–DNA hybridizations between the two strains and with closely related type strains of species of the genus *Alcaligenes* confirmed that both isolates belong to a single novel species within the genus *Alcaligenes*. On the basis of phylogenetic analyses, physiological,

The DDBJ/EMBL/GenBank accession numbers for strains NCCP-650^T (=LMG 28368^T = KCTC 42083^T = JCM 30216^T), NCCP-667, LMG 22996^T, DSM 13975^T, JCM 20522^T and DSM 16503^T are LC001699–LC001704 (*gyrB* gene) and AB983284–AB983289 (*nirK* gene), respectively; whereas the 16S rRNA gene accession numbers for strains NCCP-650^T and NCCP-667 are AB920828 and AB968096, respectively.

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biochemical characteristics and DNA–DNA hybridization, the isolated strains can be differentiated from established *Alcaligenes* species and thus represent a novel species, for which the name *Alcaligenes pakistanensis* sp. nov. is proposed with the type strain NCCP-650^T (=LMG 28368^T = KCTC42083^T = JCM 30216^T).

Keywords Heavy metals tolerance · Industrial effluent · *Alcaligenes pakistanensis* · *nirK* gene · *gyrB* gene

Introduction

The genus *Alcaligenes* was proposed in 1919 (Castellani and Chalmers 1919) and placed in the family *Alcaligenaceae* (De Ley et al. 1986) with the type species, *Alcaligenes faecalis*. So far, the genus *Alcaligenes* contains only two species *A. aquatilis* and *A. faecalis*, and the latter has been split into three sub species *A. faecalis* subsp. *faecalis, A. faecalis* subsp. *parafaecalis* and *A. faecalis phenolicus* (Euzéby 1997). Though the members of this genus were isolated from soil, sediment, bioprocess residues and water (Rehfuss and Urban 2005; Schroll et al. 2001; Van Trappen et al. 2005), some strains have also been reported to be isolated from clinical specimens (Busse and Auling 2005) and thus attract particular interest. This genus comprises of aerobic, motile, Gram-negative non-fermentative coccobacillary rods, which form non-pigmented colonies on nutrient agar. Some organisms were reported to have potential roles in bioremediation due to the presence of genes encoding for copper containing nitrite reductase and phenol hydroxylase (Rehfuss and Urban 2005).

Several microorganisms are reported to tolerate toxic concentrations of heavy metals (Abbas et al. 2014; Affan et al. 2009; Tripathi and Garg 2010; Tripathi et al. 2011; Zahoor and Rehman 2009). These heavy metal tolerant bacteria offer an opportunity to exploit their role in bioremediation of environments contaminated with heavy-metals. During our studies of microbial diversity for heavy-metal tolerant bacteria, strains NCCP-650^T and NCCP-667 were isolated on tryptic soy agar (TSA, BD, USA) by a dilution plate method from industrial effluent samples. The aim of this study was to delineate the taxonomic position of the isolated strains by a polyphasic taxonomic characterization. On the basis of results obtained, the strains NCCP-650^T and NCCP-667 are considered to represent a novel species of the genus *Alcaligenes*.

Materials and methods

Isolation and growth of strains

Strain NCCP-650^T was isolated from industrial effluent (water and sludge sample) collected from industrial wastewater discharge channel of an industrial area (lat/lon = "33.66N 73.05E"), Islamabad, Pakistan, whereas strain NCCP-667 was isolated from tanneries effluent (water and sludge sample) collected from a leather factory (lat/lon = "31.10N 74.45E"), Kasur, Pakistan. The strains were recovered on TSA supplemented with different concentrations of heavy-metals (Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}) by a dilution plate method. The purified strains were maintained on agar medium as well as stored in glycerol (35 %, w/v) at −80 °C, and subjected to polyphasic taxonomic characterization experiments. Type strains of closely related taxa, *Alcaligenes aquatilis* LMG 22996^T, *A. faecalis* subsp. *parafaecalis* DSM 13975^T, *A. faecalis* subsp. *faecalis* JCM 20522^T and *A. faecalis* subsp. *phenolicus* DSM 16503^T were used as reference strains in the majority of experiments under the same laboratory conditions. The characterization experiments were performed at 30 °C unless otherwise mentioned.

Heavy-metals tolerance

To demonstrate the tolerance of isolated strains to toxic concentrations of heavy metals, the strains NCCP-650^T, NCCP-667 and the reference strains were grown on TSA supplemented separately with different concentrations of heavy-metals (Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}) for 5–7 days. The concentration of

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heavy-metals (Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}) in agar media was in the range of 300–3000 ppm (in an incremental addition of 300 ppm), which were prepared using the salts: $K_2Cr_2O_7$, Na_2HAsO_4, $Pb(NO_3)_2$ and $CuSO_4{\cdot}5H_2O$, respectively.

Morphology and phenotypic characterization

Colony morphology of the isolated strains was observed on isolated colonies grown on TSA for 2 days. A phase-contrast microscope (Nikon Optiphot-2, Japan) was used to examine cells of the isolated strains grown on TSA for 24–48 h and further detailed by scanning electron microscope. For the electron microscopic analysis, cells were fixed in a 2.5 % (v/v) paraformaldehyde–glutaraldehyde mixture buffered with 0.1 M phosphate (pH 7.2) for 2 h, dehydrated in graded ethanol, substituted by isoamyl acetate and after drying at critical point sputter-coated with gold (SC502, Polaron) and observed using a scanning electron microscope (FEI Quanta 250 FEG). Gram staining was performed using commercial color (Gram-staining) kit (Cat. 55542, bio Mérieux, France) according to manufacturer's instructions. The motility of strains was determined with M medium (bio Mérieux, France) in addition to microscopy. Relation to oxygen was determined on TSA by incubation in an anaerobic chamber (Mitsubishi Gas Chemicals Co., Inc.) for 10 days.

The optimum and range of pH for growth was determined in tryptic soy broth (TSB; BD, USA) by adjusting to a range of pH 4.0–10.5 (at increment of 0.5 pH unit) and OD_{600} was monitored using a spectrophotometer (Beckman Coulter Model DU730, USA). The pH values adjusted by using buffers, HCl or Na_2CO_3 (Sorokin 2005) and were verified after autoclaving. The temperature range for growth was determined on TSA (pH 7.0) by incubating at different temperatures (3, 5, 10, 15, 20, 25, 30, 33, 37, 40, 45, 50 °C) for 6 days. Growth at various NaCl concentrations was investigated in TGE (pH 7.0), which contains (per litre): beef extract (6 g), tryptone (10 g), dextrose (2 g); agar (15 g) and supplemented with various concentration of NaCl (0–10 %; w/v), at adjusted pH 7.0, and incubated for 6 days.

Physiological and biochemical characteristics were determined using API 20E (Cat. 20100), API 20NE (Cat. 20050) and API 50CH (Cat. 50300) galleries (bio Mérieux, France). Since the strains showed mainly negative reactions for utilization of various carbon sources with API 20E, API 50CH and API 20NE, thus an extended array of metabolic features of the strains was analyzed using the Biolog GN2 characterization system (Biolog Inc. USA). Biolog test was performed by growing the strain NCCP-650^T along with reference strains on Biolog Universal Growth (BUG) agar medium according to the instructions of the manufacturer. Catalase and oxidase activities were determined by using API Color Catalase (Cat. 55561) and API Oxidase (Cat. 55635) reagents (bio Mérieux, France). Resistance to antibiotics was assessed with an ATB-VET (Cat. 14289) strip (bio Mérieux, France) and enzyme activities were determined with an API ZYM (Cat. 25200) strip (bio Mérieux, France). API suspension medium was used to inoculate the strips. All commercial kits were used according to the manufacturers' protocols.

Amplification and sequencing of 16S rRNA, *gyrase subunit B* (*gyrB*) and *nitrite reductase* (*nirK*) genes and the phylogenetic analysis

Nearly complete 16S rRNA gene sequence of the isolated strains was amplified and sequenced as previously described (Roohi et al. 2014); whereas, *gyrB* gene was amplified using the primers and PCR conditions described earlier by Brady et al. (2008). To demonstrate the presence of genes coding for copper containing nitrite reductase (NirK) and the phenol hydroxylase in the isolated strains in comparison with the reference species, PCR was performed using the primers and PCR conditions as described previously by Rehfuss and Urban (2005). All the purified PCR products were sequenced using the same primers on an ABI DNA analyzer. The sequences obtained were assembled using BioEdit software to get consensus sequence of the genes and submitted to DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The DDBJ/EMBL/GenBank accession numbers for strains NCCP-650^T (=LMG 28368^T = KCTC42083^T = JCM 30216^T), NCCP-667, LMG 22996^T, DSM 13975^T, JCM 20522^T and DSM 16503^T are LC001699–LC001704 (*gyrB* gene) and AB983284–AB983289 (*nirK* gene), respectively; whereas the 16S rRNA gene accession numbers for strains NCCP-650^T and NCCP-667 are AB920828 and AB968096, respectively.

The strain was identified using the sequence of 16S rRNA gene on Ez-Taxon Server (http://eztaxon-e.ezbiocloud.net) and BLAST search at the DDBJ/NCBI servers. The 16S rRNA gene sequences of closely related validly named type strains were retrieved from the database of the EzTaxon Server and phylogenetic trees were constructed as described previously (Ahmed et al. 2014) using three algorithms: neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) methods. The stability of the relationship was assessed with bootstrap analysis, by performing 1000 re-sampling for the tree topology. The sequence similarities of *gyrB* and *nirK* genes of isolated strains were compared with the sequences of reference strains using the Kimura 2-parameter model contained in MEGA 6 software package.

DNA base composition and DNA–DNA hybridization

For DNA G+C content analysis and DNA–DNA hybridization, genomic DNA of strains NCCP-650^T and NCCP-667 and the reference strains were isolated using Qiagen Genomic-tip 500/G following the manufacturer's protocol, with a minor modification in which RNase T1 was used in addition to RNase A. To confirm that strains NCCP-650^T and NCCP-667 belong to the same species, DNA–DNA hybridization was performed at 45 °C with photobiotin-labelled DNA and microplates as described by Ezaki et al. (1989), using a Fluoroskan Ascent Plate Reader (Thermo Lab Systems, USA) for fluorescence measurements. To establish a separate identity of the isolated strains NCCP-650^T and NCCP-667 as a new species, DNA–DNA hybridization between strain NCCP-650^T and with the reference strains, *Alcaligenes aquatilis* LMG 22996^T, *A. faecalis* subsp. *parafaecalis* DSM 13975^T, *A. faecalis* subsp. *faecalis* JCM 20522^T and *A. faecalis* subsp. *phenolicus* DSM 16503^T, were performed with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the means of the remaining three values were converted to percentage DNA–DNA relatedness values.

To determine DNA G+C contents, the genomic DNA was digested with P1 nuclease and alkaline phosphatase. The DNA G+C contents were analyzed by HPLC (model UFLC, Shimadzu, Japan) at 270 nm using solvent $NH_4H_2PO_4$ (0.02 M) –CH_3CN (v/v 20:1) with Cosmosil 5C18 column (4.6 by 150 mm; Nacalai Tesque, Japan; reversed phase silica gel; C18).

Chemotaxonomic analyses

For cellular fatty acids analysis, the isolates and the reference strains were grown on TSA for 24 h. The cellular fatty acid methyl esters were prepared (Sasser 1990) and were analyzed by GC (6890; Hewlett Packard) according to the standard protocol of the Sherlock Microbial Identification System (MIDI Sherlock version 4.5, MIDI database TSBA40 4.10). Respiratory quinone and polar lipids of strain NCCP-650^T and the closely related reference strains were extracted and analyzed from 100 mg lyophilized cells grown in PYE (0.3 % peptone from casein, 0.3 % yeast extract, pH 7.2) for 24 h as described by Tindall (1990a, b) and Altenburger et al. (1996). Polyamines were extracted and analyzed from biomass grown in PYE medium as described by Busse and Auling (1988). For HPLC analysis, conditions were applied reported by Busse et al. (1997). HPLC equipment applied for analyses of quinones and polyamines was reported by Stolz et al. (2007). Strain NCCP-650^T along with the reference species were analysed for whole cell sugars using lyophilized cells as described by Staneck and Roberts (1974) with the modification that sugars were identified on HPLC instead of TLC (Mikami and Ishida 1983).

Results and discussion

The isolated strains, NCCP-650^T and NCCP-667, formed off-white colonies, which were circular, low-convex with smooth surface and older colonies spread with irregular margins on TSA medium. Cells of the strains were Gram-stain negative, aerobic, motile short rods, mostly occurring in pairs, sometimes in single and rarely in quadrant form (Fig. 1). Cells grew at pH range of 5.5–10.0 (optimal at pH 6.5–7.5) and showed no growth at pH 5.0 or 10.5. The strains tolerated 0–7 % NaCl (w/v) (optimum 0–1 %) in TSB medium. The growth of the strains was observed at temperature range of 10–37 °C (optimal growth at 25–33 °C); no growth was observed at 40 °C after 6 days, which differentiated strain NCCP-650^T from all the reference species that exhibited growth at

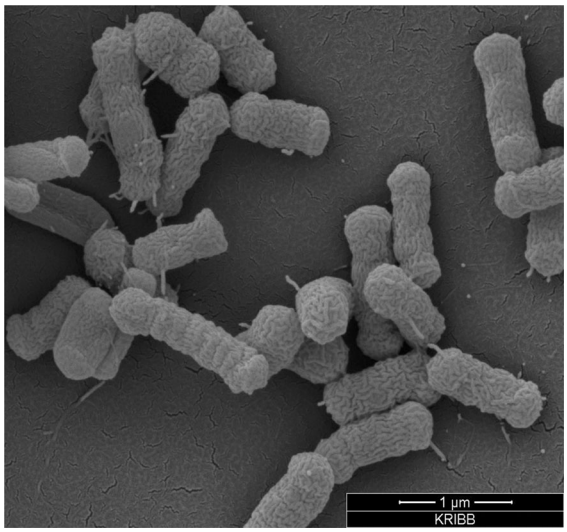

Fig. 1 Scanning electron micrograph of cells of *Alcaligenes pakistanensis* NCCP-650^T grown on tryptic soy agar medium at 30 °C for 48 h

40–42 °C with optimum growth at 33–37 °C (Rehfuss and Urban 2005). Characterization by API 50CHB, API 20E and API 20NE kits produced mostly negative results (except positive results for acetoin production, citrate utilization and assimilation of capric acid, malate, trisodium citrate and phenyl acetic acid) and were similar to those of the closely related reference species; however, several physiological (mainly obtained in Biolog GN2 characterization) and some biochemical characteristics also differentiated the novel strains from the reference species (detailed results are provided in Table 1 and in the species description). Both their isolates were overall similar in the phenotypic features but some variable results were also obtained in Biolog tests and API ATB-Vet for analysis of resistance to antibiotics (Table 1).

The two isolates, NCCP-650^T and NCCP-667 showed tolerance to toxic concentrations of heavy metals, including Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}. They grew well on TSA medium containing Cr 1500 ppm, As 3000 ppm, Pb 2100 ppm and Cu 1800 ppm. In comparison, the closely related reference strains were also tested for tolerance to heavy-metals under similar conditions. It was found that the isolated strains differed for tolerance to Cr^{+2} and Pb^{+2} from the closely related reference species (Table 1), however, no difference was observed for tolerance to Cu, As and boron when compared with the reference species, suggesting that the members of genus *Alcaligenes* are mostly tolerant to heavy metals. Compared with the previously reported heavy-metal tolerant bacteria (Abbas et al. 2014; Affan et al. 2009; Tripathi and Garg 2010; Tripathi et al. 2011; Zahoor and Rehman 2009), the strains NCCP-650^T and NCCP-667 can be considered as highly tolerant to toxic concentrations of heavy-metals.

Phylogenetic analysis, DNA–DNA hybridization and DNA base composition

Comparative sequence analyses of the 16S rRNA, *gyrB* and *nirK* genes were used to confirm the inter species relatedness of isolated strains NCCP-650^T and NCCP-667 with type strains of closely related reference species. Sequence comparison of the 16S rRNA genes of strains NCCP-650^T and NCCP-667 were carried out using Ez-Taxon Server database (http://www.ezbiocloud.net/). The 16S rRNA gene sequence of strain NCCP-650^T showed the highest similarity (98.79 %) with *A. aquatilis* LMG 22996^T (GenBank accession no. JX986976); the similarity values with other established species of the genus *Alcaligenes* were 98.76–98.22 %. The 16S rRNA gene sequence similarity between the isolated strains was 99.22 %. A neighbor-joining phylogenetic tree constructed based on a comparison of 1321 aligned nucleotides (without gaps and ambiguous nucleotides) showed that both isolates formed a coherent unit at a high bootstrap value (95 %) within the clade comprising species of genus *Alcaligenes* (Fig. 2). The nodes of this clade also appeared with the same species at high bootstrap values, when phylogenetic trees were constructed using maximum-likelihood and maximum parsimony algorithms (Supplementary Fig 2 & 3), suggesting a strong affiliation of the isolated strains NCCP-650^T and NCCP-667 with the established species of the genus *Alcaligenes*. The sequence similarity values of housekeeping genes *gyrB* and *nirK* of the isolates NCCP-650^T and NCCP-667 were 100 % with each other, respectively; however, there were notably low similarity values (89.1–85.1 and 92.4–89.7 %, respectively) with the species of genus *Alcaligenes*. The analysis based on deduced amino acid sequences of the *gyrB* gene of the isolates showed 95.2–89.7 % similarity with the reference species (the highest similarity with *A. faecalis* subsp. *parafaecalis* DSM 13975^T).

Table 1 Characteristics that differentiate novel strain NCCP-650^T from the type strains of closely related species of the genus *Alcaligenes*

Characteristics	1	2	3	4	5
Growth at temperature (°C) range (optimum)	10–37 (25–33)	10–40 (25–33)	10–40 (33–37)	10–40 (33–37)	10–40 (33–37)
Tolerance to heavy-metal					
Chromium (ppm)	1500	900	900	900	900
Lead (ppm)	2100	1800	1800	1800	1800
Tryptophane deaminase	−	+	−	+	−
Oxidation/reduction of substrate: (Biolog)					
Dextrin	−	−	−	−	+
Glycogen	−	w	−	w	+
Tween 40	v	−	−	+	+
Tween 80	v	+	−	+	+
D-Fructose	v	−	−	−	−
L-Fucose	v	−	−	−	−
D-Galactose	v	−	−	−	−
Maltose	v	−	−	−	w
D-Mannitol	v	−	−	−	w
D-Mannose	v	−	−	−	+
γ-Hydroxy butyric acid	+	−	+	+	+
α-Keto butyric acid	−	+	−	+	+
Succinamic acid	+	+	−	w	+
L-Alanyl-glycine	−	−	−	−	+
L-Asparagine	+	−	−	+	+
L-Aspartic acid	v	+	−	+	+
Glycyl-L-aspartic acid	−	−	−	−	+
Glycyl-L-glutamic acid	−	−	−	−	+
L-Histidine	w	−	−	+	+
Hydroxy-L-proline	−	−	−	−	+
L-Pyroglutamic acid	−	+	+	w	+
D-Serine	+	−	−	−	+
L-Serine	w	−	−	−	+
L-Threonine	+	+	−	w	+
D,L-Carnitine	−	−	−	−	+
γ-Amino butyric acid	−	−	−	−	+
Urocanic acid	+	−	−	−	+
Phenylethylamine	+	+	+	+	−
D,L-α-Glycerol phosphate	−	−	−	−	+
Glucose-1-phosphate	−	−	−	−	+
Glucose-6-phosphate	−	−	−	−	+
Enzyme activity (API-Zym)					
Alkaline phosphatase	++	−	+++	+	+++
Esterase (C 4)	+	w+	++	++	++
Esterase lipase (C 8)	+	w+	w+	+	++
Resistance to (μg mL^{-1})					
Amoxycillin (4)	v	R	R	R	R

Table 1 continued

Characteristics	1	2	3	4	5
Amox-clav.acid (4/2)	S	R	R	R	R
Cefoperazon (4)	S	S	R	S	R
Streptomycin (8)	R	S	R	R	R
Kanamycin (8)	S	S	R	S	R
Gentamicin (4)	S	S	R	S	S
Apramycin (16)	v	R	R	S	R
Chloramphenicol (8)	S	R	R	R	R
Sulfamethizol (100)	S	S	S	S	wR
Flumequine (4)	S	S	R	S	R
Oxolinic acid (2)	S	S	S	S	R
Whole cell sugars (molar ratio, %)	Rib (80)	Rib (95)	Rib (96)	Rib (100)	Rib (73)
	Man (15)	Glu (5)	Glu (4)		Gal (13)
	Glu (5)				Man (10)
					Glu (4)
G+C content, mol%	55.5	56[a]	56[a]	56–59[a]	54.8[a]

All data are from this study unless otherwise mentioned

Strains: 1, strain NCCP-650^T/NCCP-667; 2, *A. aquatilis* LMG 22996^T; 3, *A. faecalis* subsp. *parafaecalis* DSM 13975^T; 4, *A. faecalis* subsp. *faecalis* JCM 20522^T; 5, *A. faecalis* subsp. *phenolicus* DSM 16503^T

+++ very strongly positive, ++ strongly positive, + positive, *w*+ weakly positive, − negative, *v* variable results between the strains, *R* resistant to the antibiotic, *wR* weakly resistant, *S* sensitive, *Rha* rhamnose, *Rib* ribose, *Glu* glucose, *Man* mannose, *Gal* galactose

[a] Data from previous studies (Rehfuss and Urban 2005; Schroll et al. 2001; Van Trappen et al. 2005)

A single PCR product of the *nirK* gene (expected size of ~470 bp) was amplified, but no amplicon (expected size ~700 bp) of phenol hydroxylase (LmPH) gene was visualized on an ethidium bromide gel for the isolated strains. The similarities of deduced amino acids for *nirK* gene of the isolates were 99.10–97.26 % with *A. faecalis*, but low similarity (95.39 %) was observed with *A. aquatilis* LMG 22995^T. Both the isolates showed 100 % similarity of deduced amino acids sequences of *nirK* and *gyrB* genes, suggesting that these belong to the same species.

In summary, the analyses of 16S rRNA, *gyrB* and *nirK* gene sequences showed that the strains NCCP-650^T and NCCP-667 are closely related to the members of genus *Alcaligenes*; however, based on physiological and biochemical features (Table 1), they are also distinct from all the recognized species of this genus. Although 16S rRNA gene sequences of the isolates exhibited greater than 97 % similarity with the members of genus *Alcaligenes*, the findings of the low sequence similarity of housekeeping gene sequences for *gyrB* and *nirK* supported the hypothesis that these strains belong to a novel species. To confirm this hypothesis, DNA–DNA hybridization analysis was also performed. It was found that the DNA–DNA relatedness between both the isolated strains NCCP-650^T and NCCP-667 was 93.6 (±3.1) %, confirming that these strains belong to the same species. However, DNA–DNA hybridization values between NCCP-650^T and the reference strains, *Alcaligenes faecalis* subsp. *parafaecalis* DSM 13975^T, *A. faecalis* subsp. *phenolicus* DSM 16503^T, *A. faecalis* subsp. *faecalis* JCM 20522^T and *A. aquatilis* LMG 22996^T were determined to be 39.8 (±4.1), 34.7 (±4.7), 31.6 (±5.5) and 10.8 (±3.2) %, respectively. These values are clearly below the threshold value of 70 %, demonstrating that the two isolates are representatives of a novel species (Wayne et al. 1987). The DNA G+C content of strain NCCP-650^T was 55.5 mol% as determined by HPLC. These results are consistent with members of the genus as previously reported (Rehfuss and Urban 2005; Van Trappen et al. 2005), which support affiliation of the isolates to the genus *Alcaligenes*.

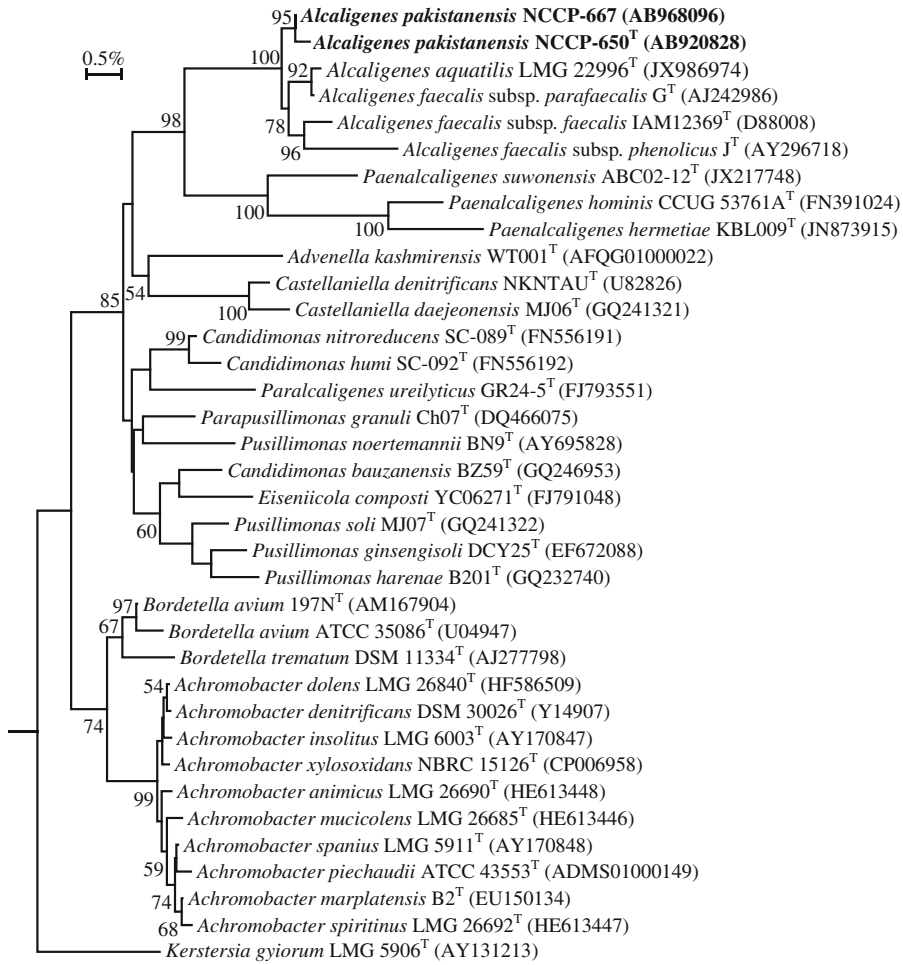

Fig. 2 Neighbour-joining phylogenetic tree inferred from 16S rRNA gene sequence showing inter-relationship of strain NCCP-650^T with the closely related species of the genus *Alcaligenes* and other related genera. Data with gaps and ambiguous nucleotides were removed during alignment for the construction of tree, which was generated using the MEGA 6.0 software package (Tamura et al. 2013) based on a comparison of 1321 nucleotides and was rooted by using *Kerstersia gyiorum* LMG 5906^T (AY131213) as an out-group. Bootstrap values (only >50 % are shown), expressed as a percentage of 1000 replications, are given at the branching points. *Bar* 0.5 % sequence divergence. The accession number of each type strain is shown in parentheses

Chemotaxonomic analysis

The cellular fatty acid profile of the isolated strains consisted predominantly of summed features 3 ($C_{16:1}$ ω7c/iso-$C_{15:0}$ 2-OH as defined by MIDI) and $C_{16:0}$ followed by Summed features 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3-OH), $C_{17:0}$ Cyclo, $C_{18:1}$ ω7*c*, $C_{12:0}$ 2-OH, $C_{14:0}$ and $C_{10:0}$ and other minor components (Table 2). Major components of this profile are similar to those found in other members of the genus, albeit some variation in values of these components clearly differentiated our strains from the closely related reference species of the genus. The presence of summed features 3, $C_{16:0}$, summed features 2 and $C_{17:0}$ Cyclo as major components has been observed in members of the *Alcaligenaceae* (Coenye et al. 2003; Vandamme et al. 1995, 1996).

Ubiquinone Q-8 was observed in strain NCCP-650^T as the major component of respiratory lipoquinone. Ubiquinone Q-8 was also detected in all the reference species. Previously, the type strain of the reference species, *A. faecalis* subsp. *parafaecalis* DSM 13975^T was reported to contain ubiquinone Q-8 system (Schroll et al. 2001). Our results are in agreement

Table 2 Cellular fatty acid profiles (%) of strain NCCP-650^T and type strains of reference species of the genus *Alcaligenes*

Characteristics	NCCP-650^T/ NCCP-667	*A. aquatilis* LMG 22996^T	*A. faecalis* subsp. *parafaecalis* DSM 13975^T	*A. faecalis* subsp. *faecalis* JCM 20522^T	*A. faecalis* subsp. *phenolicus* DSM 16503^T
$C_{10:0}$	1.1 ± 0.1	2.1 ± 0.2	2.1 ± 0.2	1.9 ± 0.3	0.1 ± 0
$C_{12:0}$	0.4 ± 0	1.0 ± 0.2	0.8 ± 0.1	1.7 ± 0.3	4.0 ± 0.2
$C_{14:0}$	1.4 ± 0.1	0.6 ± 0.1	2.3 ± 0.1	0.9 ± 0	1.1 ± 0.1
$C_{16:0}$	32.4 ± 1.0	29.6 ± 1.5	30.6 ± 2.2	29.9 ± 2.9	31.5 ± 0.9
$C_{17:0}$	0.2 ± 0.1	0.5 ± 0	0.1 ± 0	0.4 ± 0	0.3 ± 0
$C_{18:0}$	0.6 ± 0.2	0.7 ± 0.1	0.4 ± 0.1	0.8 ± 0.3	0.5 ± 0
$C_{12:0}$ 2-OH	2.0 ± 0.1	2.1 ± 0.2	2.5 ± 0.3	2.6 ± 0.4	2.7 ± 0.1
$C_{16:0}$ 3-OH	0.4 ± 0	0.4 ± 0	0.5 ± 0.1	0.4 ± 0	0.3 ± 0
$C_{17:0}$ Cyclo	8.9 ± 1.6	8.2 ± 3.2	9.0 ± 0.2	8.0 ± 2.7	12.3 ± 1.0
$C_{18:1}$ ω7*c*	7.6 ± 1.3	11.0 ± 1.8	4.5 ± 0.6	8.1 ± 1.7	4.8 ± 0.6
Summed features 2[a]	9.4 ± 0.7	10.3 ± 1.3	11.4 ± 1.6	12.1 ± 2.1	11.7 ± 1.1
Summed features 3[a]	33.8 ± 2.7	31.5 ± 2.1	34.6 ± 0.9	31.3 ± 1.1	29.1 ± 1.0

All data are obtained in this study. Values (average of two readings of each strain from two independent experiments and their standard deviation) are percentages of total fatty acid detected

Those values of cellular fatty acid components were deleted if present <1 % in all the species and/or absent in some species

[a] Summed feature 2 comprised one or more of iso-$C_{16:1}$ I/$C_{14:0}$ 3OH, and Summed feature 3 comprised one or more of $C_{16:1}$ ω7c/ iso-$C_{15:0}$ 2OH, which could not have been separated by MIDI system

with those reported in *A. faecalis* subsp. *parafaecalis* DSM 13975^T and also conforms the reports that the predominant presence of Q-8 is a common trait of members of *Betaproteobacteria* including members of the *Alcaligenaceae* such as *A. faecalis* subsp. *faecalis* IAM 12586^T and *A. faecalis* subsp. *parafaecalis* DSM 13975^T (Schroll et al. 2001; Yokota et al. 1992).

The polar lipid profile of strain NCCP-650^T showed high similarity with those of the reference species (Supplementary Fig. 1). The polar lipids of strain NCCP-650^T were predominantly identified to be diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and unidentified aminolipid (AL1). Furthermore, moderate to minor amounts of phosphatidylserine (PS), another unidentified aminolipid (AL2) and six unidentified polar lipids (L1–6) without an amino residue, a phosphate residue or sugar moiety were also observed. Though the presence of polar lipid L5 distinguished NCCP-650^T from all reference species and the presence of aminolipid AL2 from all reference species except *A. faecalis* subsp. *parafaecalis* DSM 13975^T (Supplementary Fig. 1) not too much significance should be given to this observation because the amounts detected of these two lipids were rather low. Also the presence of several unidentified polar lipids in the reference species but absent in NCCP-650^T were too low to be considered as a robust distinguishing trait.

The polyamines pattern was composed of putrescine [45.9 μmol (g dry weight)$^{-1}$], spermidine [2.2 μmol (g dry weight)$^{-1}$] and traces of cadaverine and spermine [<0.1 μmol (g dry weight)$^{-1}$]. The absence of any detectable 2-hydroxyputresence is very rarely observed among *Betaproteobacteria* but the close relative of NCCP-650^T, *A. faecalis* subsp. *parafaecalis* DSM 13975^T was also reported to lack 2-hydroxyputresence (Schroll et al. 2001), whereas the type species of *A. faecalis* subsp. *faecalis* was shown to contain this polyamine though in relatively low amounts (Busse and Auling 1988). However, the absence of this polyamine in both NCCP-650^T and *A. faecalis* subsp. *parafaecalis* reflects the close relatedness between the two (Fig. 2).

Ribose was found to be the major whole cell sugar in strain NCCP-650^T and the reference species, whereas mannose (15 %), and glucose (5 %) were detected as minor components in strain NCCP-650^T. The comparison of molar ratio of sugars in all the strains is presented in Table 1. Mannose was absent in *A. aquatilis* LMG 22996^T, *A. faecalis* subsp.

parafaecalis DSM 13975^T and *A. faecalis* subsp. *faecalis* JCM 20522^T, whereas a significant amount (13 %) of galactose was detected in *A. faecalis* subsp. *phenolicus* DSM 16503^T (Table 1). Our data demonstrate that the genus *Alcaligenes* is heterogeneous in terms of minor whole cell sugars.

On the basis of phenotypic, genotypic, chemotaxonomic data and phylogenetic analyses, both the isolated strains NCCP-650^T and NCCP-667 belong to a single novel species of the genus *Alcaligenes*, for which the name *Alcaligenes pakistanensis* sp. nov., is proposed with the type strain NCCP-650^T and its description is given below:

Description of *Alcaligenes pakistanensis* sp. nov.

Alcaligenes pakistanensis (pa.kis.tan.en'sis. N.L. masc. adj. *pakistanensis* from Pakistan, where the organism was isolated).

Cells are Gram-stain negative, strictly aerobic, motile and short rods, mostly occur in pairs, sometimes in single and rarely occur in quadrant form. Colonies are off-white in color, circular, low-convex with smooth surface; older colonies spread with irregular margins on TSA medium. Cells grow at temperature range of 10–37 °C (optimal growth at 25–33 °C), pH range of 5.5–10.0 (optimal at pH 6.5–7.5) and in 0–7 % NaCl (w/v) (optimum 0–1 %) in TSB medium. Tolerant to heavy-metals (i.e. Cr^{+2}, As^{+2}, Pb^{+2} and Cu^{+2}). Possesses the copper containing nitrite reductase *nirK* gene. Catalase and oxidase activities are positive. Positive for Voges–Proskauer reaction and citrate utilization but negative for nitrate reduction to N_2, indole production, lysine & ornithine decarboxylases, arginine dihydrolase, tryptophane deaminase, β-galactosidase (2-nitrophenyl-β-D-galactopyranoside) and H_2S production. Gelatin, urea and esculin are not hydrolyzed. Can assimilate capric acid, trisodium citrate, phenyl acetic acid and malate but not glucose, mannose, mannitol, arabinose, potassium gluconate, adipic acid and maltose. No oxidation/fermentation of D-glucose, D-sorbitol, amygdalin, L-arabinose, inositol, L-rhamnose, D-sucrose, D-melibiose, and D-mannitol. No acid is produced from substrates in the API-50CH system (bio Mérieux, France). Strong enzyme activity observed for acid phosphatase, leucine arylamidase, alkaline phosphatase, esterase (C 4), esterase lipase (C 8), valine arylamidase, naphthol-As-BI-phosphohydrolase, but weak enzyme activity for lipase (C 14), cystine arylamidase, trypsin, α-chymotrypsin, whereas no enzyme activity for α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase (API-Zym, bio Mérieux, France). The following compounds are used as sole carbon sources as determined by Biolog GN plates: methyl pyruvate, cis-aconitic acid, citric acid, formic acid, α-hydroxy butyric acid, β-hydroxy butyric acid, γ-hydroxy butyric acid, *p*-hydroxy phenylacetic acid, D,L-lactic acid, malonic acid, propionic acid, succinic acid, bromo succinic acid, succinamic acid, L-alaninamide, D-alanine, L-alanine, L-asparagine, L-glutamic acid, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-proline, D-serine, L-serine, L-threonine, urocanic acid, phenylethylamine, mono-methyl-succinate (weak), acetic acid (weak), whereas variable results for Tween 40, Tween 80, L-arabinose, D-arabitol, D-fructose, L-fucose, D-galactose, maltose, D-mannitol, D-mannose, L-alanyl-glycine, L-aspartic acid, but the following substrates are not used as carbon source: dextrin, glycogen, D-cellobiose, L-erythritol, α-D-lactose, lactulose, α-keto butyric acid, α-keto glutaric acid, α-keto valeric acid, quinic acid, D-saccharic acid, sebacic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, hydroxy-L-proline, L-pyroglutamic acid, D,L-carnitine, γ-amino butyric acid, inosine, putrescine, D,L-α-glycerol phosphate, glucose-1-phosphate, glucose-6-phosphate. Major polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, and an unidentified aminolipid (AL1). Moderate to minor amounts of phosphatidylserine, one unidentified aminolipid (AL2) and six unidentified polar lipids (L1–6). The polyamine pattern contains the major compound putrescine and moderate amounts of spermidine. 2-hydroxyputrescine is absent. Predominant cellular fatty acids are summed features 3 ($C_{16:1}$ ω7c/iso-$C_{15:0}$ 2OH as defined by MIDI) and $C_{16:0}$ followed by Summed features 2 (iso-$C_{16:1}$ I/$C_{14:0}$ 3OH), $C_{17:0}$ Cyclo, $C_{18:1}$ ω7*c*, $C_{12:0}$ 2-OH, $C_{14:0}$ and $C_{10:0}$. The major quinone is ubiquinone Q-8. The DNA G+C content of the type strain is 55.5 mol%.

Strain NCCP-650^T (=LMG 28368^T = KCTC42083^T = JCM 30216^T) is the type strain, isolated from an industrial effluent (water and sludge) sample collected from Industrial waste water discharge channel of Sector I-9 Industrial area, Islamabad, Pakistan.

The DDBJ/EMBL/GenBank accession numbers for the type strain NCCP-650^T are AB920828 (16S rRNA

gene), LC001699 (*gyrB* gene) and AB983284 (*nirK* gene).

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References

Abbas S, Ahmed I, Kudo T, Iida T, Ali GM, Fujiwara T, Ohkuma M (2014) Heavy metal-tolerant and psychrotolerant bacterium *Acinetobacter pakistanensis* sp. nov., isolated from a textile dyeing wastewater treatment pond. Pak J Agri Sci 51:595–608

Affan Q-u-A, Shoeb E, Badar U, Akhtar J (2009) Isolation and characterization of bacterial isolates having heavy metal tolerance. J Basic Appl Sci 5:55–60

Ahmed I, Sin Y, Paek J, Ehsan M, Hayat R, Iqbal M, Chang YH (2014) Description of *Lysinibacillus pakistanensis*. Int J Agric Biol 16:447–450

Altenburger P, Kämpferb P, Makristathisc A, Lubitza W, Bussea H-J (1996) Classification of bacteria isolated from a medieval wall painting. J Biotechnol 47:39–52. doi:10.1016/0168-1656(96)01376-4

Brady C, Cleenwerck I, Venter S, Vancanneyt M, Swings J, Coutinho T (2008) Phylogeny and identification of *Pantoea* species associated with plants, humans and the natural environment based on multilocus sequence analysis (MLSA). Syst Appl Microbiol 31:447–460. doi:10.1016/j.syapm.2008.09.004

Busse J, Auling G (1988) Polyamine pattern as a chemotaxonomic marker within the *Proteobacteria*. Syst Appl Microbiol 11:1–8. doi:10.1016/S0723-2020(88)80040-7

Busse H-J, Auling G (2005) Genus I. *Alcaligenes*. Castellani and Chalmers 1919, 936AL. In: Brenner DJ, Krieg NR, Staley JT (eds) Bergey's manual of systematic bacteriology, vol 2, 2nd edn. Springer, USA, pp 653–658

Busse H-J, Bunka S, Hensel A, Lubitz W (1997) Discrimination of members of the family *Pasteurellaceae* based on polyamine patterns. Int J Syst Bacteriol 47:698–708. doi:10.1099/00207713-47-3-698

Castellani A, Chalmers AJ (eds) (1919) Manual of tropical medicine, 3rd edn. Williams Wood and Co., New York

Coenye T, Vancanneyt M, Cnockaert MC, Falsen E, Swings J, Vandamme P (2003) *Kerstersia gyiorum* gen. nov., sp. nov., a novel *Alcaligenes faecalis*-like organism isolated from human clinical samples, and reclassification of *Alcaligenes denitrificans* Ruger and Tan 1983 as *Achromobacter denitrificans* comb. nov. Int J Syst Evol Microbiol 53:1825–1831. doi:10.1099/ijs.0.02609-0

De Ley J, Segers P, Kersters K, Mannheim W, Lievens A (1986) Intra-and intergeneric similarities of the *Bordetella* ribosomal ribonucleic acid cistrons: proposal for a new family, *Alcaligenaceae*. Int J Syst Bacteriol 36:405–414. doi:10.1099/00207713-36-3-405

Euzéby JP (1997) List of bacterial names with standing in nomenclature: a folder available on the Internet (http://www.bacterio.net). Int J Syst Bacteriol 47:590–592 doi:10.1099/00207713-47-2-590

Ezaki T, Hashimoto Y, Yabuuchi E (1989) Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. Int J Syst Bacteriol 39:224–229. doi:10.1099/00207713-39-3-224

Mikami H, Ishida Y (1983) Post-column fluorometric detection of reducing sugars in high performance liquid chromatography using arginine. Bunseki Kagaku 32:E207–E210

Rehfuss M, Urban J (2005) *Alcaligenes faecalis* subsp. *phenolicus* subsp. nov. a phenol-degrading, denitrifying bacterium isolated from a graywater bioprocessor. Syst Appl Microbiol 28:421–429. doi:10.1016/j.syapm.2005.03.003

Roohi A, Ahmed I, Khalid N, Iqbal M, Jamil M (2014) Isolation and phylogenetic identification of halotolerant/halophilic bacteria from the salt mines of Karak, Pakistan. Int J Agric Biol 16:564–570

Sasser M (1990) Identification of bacteria by gas chromatography of cellular fatty acids. MIDI Technical Note 101

Schroll G, Busse HJ, Parrer G, Rolleke S, Lubitz W, Denner EB (2001) *Alcaligenes faecalis* subsp. *parafaecalis* subsp. nov., a bacterium accumulating poly-beta-hydroxybutyrate from acetone-butanol bioprocess residues. Syst Appl Microbiol 24:37–43. doi:10.1078/0723-2020-00001

Sorokin DY (2005) Is there a limit for high-pH life? Int J Syst Evol Microbiol 55:1405–1406. doi:10.1099/ijs.0.63737-0

Staneck JL, Roberts GD (1974) Simplified approach to identification of aerobic *Actinomycetes* by thin-layer chromatography. Appl Environ Microbiol 28:226–231

Stolz A, Busse HJ, Kampfer P (2007) *Pseudomonas knackmussii* sp. nov. Int J Syst Evol Microbiol 57:572–576. doi:10.1099/ijs.0.64761-0

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729. doi:10.1093/molbev/mst197

Tindall B (1990a) A comparative study of the lipid composition of *Halobacterium saccharovorum* from various sources. Syst Appl Microbiol 13:128–130. doi:10.1016/S0723-2020(11)80158-X

Tindall B (1990b) Lipid composition of *Halobacterium lacusprofundi*. FEMS Microbiol Lett 66:199–202. doi:10.1111/j.1574-6968.1990.tb03996.x

Tripathi M, Garg S (2010) Studies on selection of efficient bacterial strains simultaneously tolerant to hexavalent chromium and penta chlorophenol isolated from treated tannery effluent. Res J Microbiol 5:707–716

Tripathi M, Vikram S, Jain RK, Garg S (2011) Isolation and growth characteristics of chromium(VI) and pentachlorophenol tolerant bacterial Isolate from treated tannery effluent for its possible use in simultaneous bioremediation. Ind J Microbiol 51:61–69. doi:10.1007/s12088-011-0089-2

Van Trappen S, Tan TL, Samyn E, Vandamme P (2005) *Alcaligenes aquatilis* sp. nov., a novel bacterium from sediments of the Weser Estuary, Germany, and a salt marsh on Shem Creek in Charleston Harbor, USA. Int J Syst Evol Microbiol 55:2571–2575. doi:10.1099/ijs.0.63849-0

Vandamme P et al (1995) *Bordetella hinzii* sp. nov., isolated from poultry and humans. Int J Syst Bacteriol 45:37–45. doi:10.1099/00207713-45-1-37

Vandamme P et al (1996) *Bordetella trematum* sp. nov., isolated from wounds and ear infections in humans, and reassessment of *Alcaligenes denitrificans* Rüger and Tan 1983. Int J Syst Bacteriol 46:849–858. doi:10.1099/00207713-46-4-849

Wayne LG et al (1987) Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int J Syst Bacteriol 37:463–464. doi:10.1099/00207713-37-4-463

Yokota A, Akagawa-Matsushita M, Hiraishi A, Kataya-Ma Y, Urakami T, Yamasato K (1992) Distribution of quinone systems in microorganisms: gram-negative eubacteria. Bull JFCC 8:136–171

Zahoor A, Rehman A (2009) Isolation of Cr(VI) reducing bacteria from industrial effluents and their potential use in bioremediation of chromium containing wastewater. J Environ Sci 21:814–820. doi:10.1016/s1001-0742(08)62346-3