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# Phylogenetic analysis of different isolates of *Sulfobacillus* spp. isolated from uranium-rich environments and recovery of genes using integron-specific primers

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**Abstract** The isolation and phylogenetic characterization of acidophilic moderate thermophilic bacteria from different locations of uranium mines and a uranium processing mill in Pakistan is reported. The dominant culturable bacteria found were related to *Sulfobacillus thermosulfidooxidans* in all the samples analyzed. Different strains displayed different levels of identity (95–97%) to16S rDNA of known strains of this species, indicating group heterogeneity. Genomic DNA from five isolates was subjected to amplification using integron-specific primers HS286 and HS287. Recovery of different integron-linked genes from one of the isolates indicated the usefulness of this approach for gene mining in place of traditional gene recovery methodologies.

**Keywords** Acidophilic moderate thermophiles · Gene cassettes · Integrons · 16S rDNA analysis · Uranium rich environments

## Introduction

The interest in the study of microbial diversity has been enhanced by the fact that only a small proportion of the microbial community is culturable. This has stimulated the development and use of new molecular methods for detecting taxon-specific genes without the need for culture (Lane 1991).

The microbial genomic era has revolutionized our understanding of the composition of bacterial gene pools and how different genes have been transferred vertically as well as horizontally. It has been clearly demonstrated that a substantial part of the bacterial gene pool is extrachromosomal and can vary between members of the same species. In addition, a large proportion of bacterial genes has been acquired by horizontal gene transfer (Ochman et al. 2000). Horizontal gene transfer in bacteria is facilitated by a number of genetic elements including phages, plasmids, transposons, and integrons. Previously, most attention has been focused on plasmids and transposons. This is particularly true for environmental microorganisms (Smalla et al. 2000). Therefore, one possible way to recover a proportion of the environmental gene pool is to target conserved genetic sequences associated with mobile genetic elements.

An integron is defined as a genetic element that possesses a site, *attI*, at which additional DNA, in the form of gene cassettes, can be integrated by site-specific recombination, and which encodes an enzyme, integrase, that mediates these site-specific recombination events (Bennett 1999). Gene cassettes are discrete genetic elements that may exist as free, circular, non-replicating DNA molecules when moving from one genetic site to another (Collis and Hall 1992), but which are normally found as linear sequences that constitute part of a larger DNA molecule, such as a plasmid or chromosome. Gene cassettes normally contain only a single gene and an additional short sequence, called a 59-base element, that functions as a specific recombination site (Hall et al. 1991). The genes carried on gene cassettes usually lack a promoter and are expressed from a promoter on the integron (Hall and Collis 1995). The range of genera now known to host integrons is very broad and includes *Vibrio*, *Pseudomonas*, *Shewanella*, *Geobacter*, *Xanthomonas*, *Listonella*, *Photobacterium*,

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*Moritella*, *Alteromonas*, *Treponema*, *Nitrosomonas*, and *Acidithiobacillus* (Rowe-Magnus and Mazel 2001).

Microbial biodiversity in the metal-rich effluent at active and derelict mines, mine spoils, and tailing heaps has not been fully explored although metal recovery activities employing biohydrometallurgical techniques are quite widespread throughout the world. Mineral leaching environments are dominated by groups of bacteria, which are mostly acidophilic in nature (Johnson 1998). Three main groups of acidophiles that are active at and around 50 °C have been characterized of which two are comprised of bacteria while one group belongs to the archaea (Norris and Johnson 1998). The acidophile most readily isolated from hot spring runoff, coal spoil heaps, and acidic water at mine sites in many countries are gram-positive *Sulfobacillus* species (Norris and Johnson 1998). These bacteria are able to grow heterotrophically, mixotrophically, and autotrophically. This study reports the presence of such microbial types in uranium-rich environments in Pakistan, which have not been previously investigated. Moreover, the presence of integrons, which can capture different types of genes, is reported for the first time in such bacteria.

## Materials and methods

### Origin and enrichment of acidophilic moderate thermophilic bacteria

Different environmental samples were collected in sterile plastic bottles from different locations of uranium mines and a uranium-processing mill in Pakistan (Table 1). Among five environmental samples collected, three solid samples were made into slurries by mixing with sterile water (50%, w/v). All the samples were plated (in triplicate) on selective solid media (Johnson 1995) at 45 °C. Isolated colonies appeared after 4–5 days of incubation, which were subcultured in a liquid version of the above media for enrichment. Growth was confirmed by monitoring the oxidation of ferrous sulfate, which was used as an energy source in these media.

### Optimization of growth-affecting parameters

The optimum temperature for growth was obtained by growing bacteria in liquid medium (Johnson 1995) at four different temperatures, i.e., 30, 45, 50 and 60 °C, and growth was compared by estimating the rate of ferrous iron (50 mM) oxidation (data not shown). Optimum pH for ferrous iron oxidation was determined by growing them at pH values ranging from 1.5 to 4.

**Table 1** Description of environmental samples and bacterial isolates recovered

| Origin | Nature | pH | Isolate code |
|---|---|---|---|
| Fresh tailings plant site | Liquid | 2.0 | MT-9 |
| Fresh tailing pond | Liquid | 2.0 | MT-10 |
| Lump of ore containing coal and ferric precipitates from abandoned uranium mine | Solid | 2.0 | MT-13 |
| Tailing pond number 2 (abandoned) | Solid | 4.0 | MT-16 |
| Tailing pond number 3 (abandoned) | Solid | 3.0 | MT-17 |

### Isolation of DNA

Genomic DNA was isolated from 500 ml liquid bacterial culture using a standard kit (GenomicPrep Cells and Tissue DNA Isolation Kit; Amersham Pharmacia Biotech) and stored at −20 °C till further use.

### Estimation of G + C mol%

G + C mol% of different strains was estimated by determining the melting temperature (Tm) of genomic DNA as described by Marmur and Doty (1962).

### Phylogenetic analysis of the isolates

Polymerase chain reaction (PCR) amplification of 16S ribosomal RNA genes of isolates was carried out using the following primers: forward primer (FD1) AGAGTTTGATCCTGGCTCAG and reverse primer (rP1) ACGG(ACT)TACCTTGTTACGACTT. Two times ReadyMix PCR Master Mix (ABgene) was used. Each reaction vial contained 25 $\mu$l Master Mix and the addition of the template (1 $\mu$l) and primers (FD1 and rP1, each 1 $\mu$l) resulted in a final volume of 50 $\mu$l with nanopure water. The Master Mix contained 1.25 U *Taq* DNA polymerase, 75 mM TRIS-HCl (pH 8.8 at 25 °C), 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% (v/v) Tween 20, 0.2 mM each of dATP, dCTP, dGTP, dTTP, precipitant, and red dye for electrophoresis. The reaction mixture was heated for 2 min at 96 °C and then amplification was carried out. Each cycle was comprised of 30 s at 95 °C, 40 s at 55 °C, and 2 min at 72 °C. After 30 cycles the reaction was left at 72 °C for 10 min.

The PCR product, about 1,500 bp, was purified using Qiagen commercial columns and sequenced commercially (MWG Biotech, Germany). The gene sequences were compared with others in the GenBank databases using the NCBI BLAST (www.ncbi.nlm.nih.gov). Gene sequences of 16S rDNA of selected microorganisms were obtained from GenBank and aligned with gene sequences of our isolates using CLUSTALX. The aligned sequences were used to construct a distance matrix (Jukes and Cantor 1969), after the generation of 1,000 bootstrap sets, that was subsequently used to construct a phylogenetic tree by the neighbor-joining method (Saitou and Nei 1987), using Treecon for Windows (Van de Peer and De Wachter 1993).

### Recovery of genes using integron-specific primers and PCR amplification

The primers to conserved sequences were HS286 (5′ GGGATCC TC(GC)GCT(GT)GA(GA)CGA(AC)TTGTTAG(GCA)C 3′) and HS287 (5′ GGGATCCGC(GC)GCT(GT)A(AGCT)CTC(GCA)(GA)(GA)CGTTAG(GC)C 3′). These primers target the flanking regions of 59-base element sites (Stokes et al. 2001). The underlined sequence is a *Bam*HI linker that is not complementary to 59-base element sequences. Reaction mixtures consisted of approximately 5 ng template DNA, 100 pmol of each of the primers, 200 nM deoxynucleoside triphosphate (dNTP) mix, 2 mM $MgCl_2$, and 1 U *Taq* DNA polymerase. The PCR was carried out by standard techniques with the following cycling program: 94 °C for 3 min for 1 cycle, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2.5 min for 35 cycles, and 72 °C for 5 min for 1 cycle.

### Ligation and transformation

Amplified PCR products were ligated into the pGEM-T Easy vector following the manufacturer's instructions. The ligation mixture was transformed by heat shock (42 °C) into *Escherichia*

*coli* JM109 competent cells (Promega catalog number L2001) following the manufacturer's protocol.

### Plasmid isolation

Plasmid from clones containing inserts was isolated from 3 ml overnight cultures by using the Wizard Plus Miniprep DNA purification system (Promega) as per the manufacturer's instructions.

### Selection of clones containing inserts for sequencing

Selection of clones was carried out after digesting plasmid DNA with *Eco*RI restriction enzyme. Only clones that contained observable inserts after the digestion reaction were sequenced. Dried plasmid DNA samples, approximately containing 750 ng DNA, were sent to MWG Biotech and sequenced with *M13* forward and reverse primers.

### Sequence retrieval and analysis

Sequence analyses of the cloned PCR products were performed with programs available through the National Center for Biotechnology Information (USA). BLASTN was used to search for nucleotide sequence homology. BLASTX was used to search for possible protein identities. Open reading frames (ORFs) were identified using the ORF Finder program at the National Center for Biotechnology Information.

### Nucleotide sequence accession numbers

Nucleotide sequences from partial 16S rDNA and gene cassette clones were deposited with GenBank of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). They are indicated in the text.

## Results and discussion

Phase contrast microscopic examination of cultures of these isolates showed they were all motile rod-shaped bacteria, cell length 2.0–2.5 $\mu$m, with terminal endospores. All the isolates were Gram-positive. The temperature optimum for all isolates was between 45 and 50 °C and the pH optimum for growth for all at around pH 2. The G + C mol% content of the different isolates all fell in the range 48–50 mol%.

This preliminary characterization showed that probably all the isolates belonged to the same taxon, therefore only isolates MT-13, MT-16, and MT-17 which showed slightly different patterns of iron oxidation profile (data not shown) were processed for further 16S rRNA gene sequencing and phylogenetic tree analysis. Isolates MT-13 (GenBank AY179182), MT-16 (GenBank AY179183), and MT-17 (GenBank AY179184) showed 95%, 96%, and 97% identity, respectively, to *Sulfobacillus thermosulfidooxidans* on the basis of 16S rRNA gene sequence, respectively. Similarly, they occupied a very close position to this species in a phylogenetic tree (Fig. 1).

To recover cassette-associated bacterial genes from the five strains related to *Sulfobacillus thermosulfidooxidans*, integron-specific primers HS286 and HS287 were used. PCR amplification gave positive results in only one isolate, i.e., MT-16 with multiple product fragments ranging from approximately 300 to 1,300 bp (Fig. 2).

The PCR products were cloned into pGEM T-Easy vector. Twenty-four clones were picked at random from hundreds of transformed clones. On the basis of restriction fragment length polymorphism analysis following restriction with *Eco*RI six different clones were identified. Two clones contained very small inserts and were not further characterized. Four clones contained DNA sequences greater than 700 nucleotides, (Table 2). Sequence analysis identified two unrelated sequences, a unique sequence mt-11, and mt-3, mt-4, and mt-20 (the mt-3 group) that were highly related to each other. BLASTN analysis, not included, showed identities to other integron cassettes because of the presence of the primer sequences. When this was repeated with the primer sequences removed, no closely related sequences were identified in the non-redundant data base. BLASTX and the ORF cognitor program associated with ORF Finder identified very significant similarities between the mt-3 group and the ABC-type multidrug/protein/lipid transport system (ATPase component) proteins, and significant similarities between mt-11 and an ATP-dependent exoDNase (exonuclease V) beta subunit (Table 2). It is worthy of note that the closest matches are proteins from bacteria that are phylogenetically very distant from *Sulfobacillus*.

The defining feature of a gene cassette is a 59-base element recombination site. We anticipated some clones

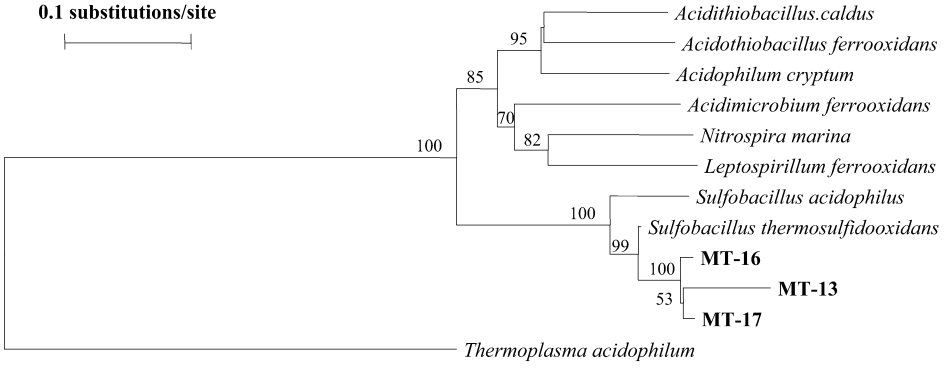


**Fig. 1** Rooted phylogenetic tree showing the relationship of the new acidophilic moderate thermophiles (in *bold*) to other acidophilic bacteria. The tree was rooted with the 16S rRNA gene sequence of *Thermoplasma acidophilum*. *Scale bar* represents the number of inferred nucleotide substitutions per site. Values at nodes indicate > 50% percentage of occurrence in 1,000 bootstrapped trees

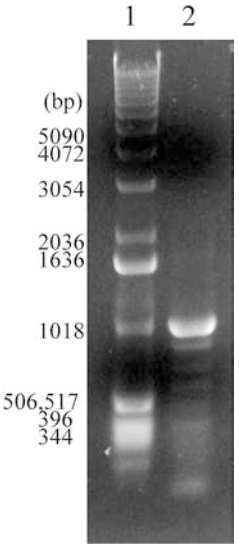


**Fig. 2** Polymerase chain reaction amplification products of genomic DNA using integron-specific HS286 and HS287 primers. *Lane 1* 1-kb DNA ladder, *lane 2* isolate MT-16

would represent multiple gene cassettes and include 59-base element sites. However, none of the amplicons contained a putative 59-base element thus suggesting that these clones represented a single cassette. Taken together, these data demonstrate that the HS286 and HS287 primer pair is highly selective for integron-associated cassette arrays.

There have been relatively few reports on the biodiversity and relative abundance of acidophilic microorganisms in mineral-leaching environments, and most have tended to focus exclusively on iron- and sulfur-oxidizing chemoautotrophs. In addition, very little is known about the microbiology of extremely acidic uranium-rich environments. Berthelot et al. (1993) found that numbers of iron-oxidizing chemolithotrophs (at $10^3$–$10^5$ $ml^{-1}$) were some two orders of magnitude greater than those of heterotrophic acidophiles in acid mine drainage (AMD) at uranium mines in Canada. Reports about the presence of acidophilic moderate thermophiles in such environments are even more sporadic. Different environmental samples collected from different locations in this study predominantly contained organisms related to *Sulfobacillus thermosulfidooxidans*. This is the first report of the isolation of bacteria related to *Sulfobacillus thermosulfidooxidans* from uranium-leaching environments. Furthermore, we have shown that at least some of these strains contain integrons and we are able to recover different genes from one such type using integron-specific primers. Consistent recovery of integron-linked gene cassettes from these bacteria over a period of 6 months suggests that laboratory adapted cultures do not readily lose integrons.

Integron-integrase-like genes have been reported in acidophilic bacteria like *Acidithiobacillus ferrooxidans* (Rowe-Magnus and Mazel 2001). This work has further broadened the range of genera know to host integrons. Integrons may be a common feature of bacteria isolated from non-clinical environments and it seems likely that integron-mediated genetic exchange can take place between acidophiles in natural habitats. The bacteria isolated in the present study represent a very distinctive group but they can clearly still host common integron-linked gene cassettes found in other unrelated bacterial groups. Such genes, which are presumably acquired through horizontal gene transfer may later follow a vertical transfer route.

Presumably, exchange or transfer of common gene cassettes between unrelated microbial groups strongly influences gene transfer in the environment.

Due to the competitive nature of PCR, the integron recovery technique itself seems likely to introduce considerable sample bias. Two factors are particularly important in this instance. The first is bias toward smaller amplicons, and the second is primer bias. The primer pair used here was designed against a database primarily composed of 59-base element sites from antibiotic-resistance gene cassettes found in class 1 integrons (Stokes et al. 2001). As the database of 59-base element sequences has expanded, it has become evident that the primer set does not encompass the sequence diversity in this family of recombination sites. Furthermore, it has been

**Table 2** Genes recovered from MT-16 using integron-specific primers HS286 and HS287. BlastX E, expected values are indicated. Open reading frame (ORF) cognitor compares the protein sequence identified by ORF Finder against the clusters of orthologous groups protein database. Accession numbers for nucleotide sequences are: mt-3 (AY179194), mt-4 (AY179195), mt-11 (AY179196), and mt-20 (AY179197). Mt3, mt4, and mt20 sequences showed high identity to each other

| Clone | Insert (bp) | Top database hit | |
|---|---|---|---|
| | | BlastX | ORF cognitor result |
| mt-3 | 1,240 | Probable ABC transporter (*Clostridium perfringens*) (NP_561296). $E = 10^{-37}$ | ABC-type multidrug/protein/lipid transport system, ATPase component |
| mt-4 | 716 | Probable ABC transporter (*Clostridium perfringens*) (NP_562441). $E = 10^{-24}$ | ABC-type multidrug/protein/lipid transport system, ATPase component |
| mt-11 | 1,077 | Hypothetical protein (*Rhodopseudomonas palustris*) (ZP_00011783). $E = 10^{-14}$ | ATP-dependent exoDNAse (exonuclease V) beta subunit (contains helicase and exonuclease domains) |
| mt-20 | 1,101 | Hypothetical protein (*Chloroflexus aurantiacus*) (ZP_00017667). $E = 10^{-42}$ | ABC-type multidrug/protein/lipid transport system, ATPase component |

suggested that 59-base element sites comprise sequence homology groups related to their origin in chromosomal integrons (Rowe-Magnus et al. 2001). The primer set used in the study may under-sample gene cassette pools, and one may anticipate that as more integron-gene cassette systems are described, new sets of primers favoring the recovery of distinct cassette pools may be designed.

Integron recovery represents a new opportunity to prospect for genes of biotechnological significance by culture-independent means. Notably, identification of gene boundaries and location in a sequence fragment is greatly simplified, the orientation of ORFs is highly predictable, and genes are prepackaged in a form amenable to manipulation by site-specific recombination. As a consequence, the overall strategy provides rapid access to a significant genetic resource in a way that is independent of prior gene sequence knowledge and recovers the gene in a form ready for direct analysis (Stokes et al. 2001).

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