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Multilocus sequence analysis of homologous recombination and diversity in *Arthrobacter sensu lato* named species and glacier-inhabiting strains

Qing Liu [a], Yu-Hua Xin [a,*], Yu-Guang Zhou [a], Wen-Xin Chen [b]

[a] China General Microbiological Culture Collection Center (CGMCC), Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, China
[b] State Key Laboratory of Agrobiotechnology, College of Biological Sciences, China Agricultural University, Beijing 100193, China

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ABSTRACT

Members of the bacterial genus *Arthrobacter sensu lato* are Gram-positive actinomycetes distributed worldwide and found in numerous environments including soil, water, glacier ice, and sewage. Homologous recombination is an important driving force in bacterial evolution, but its impact on *Arthrobacter sensu lato* evolution is poorly understood. We evaluated homologous recombination among 41 *Arthrobacter sensu lato* named species, using multilocus sequence analysis (MLSA). A high level of recombination was found, associated with strong diversification and a reticulate evolutionary pattern of *Arthrobacter sensu lato*. We also collected a total of 31 cold-adapted *Arthrobacter sensu lato* strains from two cold glaciers located in northwest China and two temperate glaciers in southwest China, and evaluated their diversity and population structure by MLSA. The glacier strains displayed high diversity, but rates of recombination among the four glacier groups were quite low, indicating that barriers to homologous recombination formed in the past among the populations on different glaciers. Our findings indicate that historical glaciation events shaped the contemporary distributions, taxonomic relationships, and phylogeographic patterns of *Arthrobacter sensu lato* species on glaciers.

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Introduction

Homologous recombination, defined as the incorporation of small similar DNA regions from a donor genome into the recipient genome, is common in prokaryotes [52], and plays an important role in the evolution and genetic diversification of bacterial lineages. Both interspecies and intraspecies recombination evidences have been found [12,19,8]. The rate of recombination is higher within species than between species, suggesting that homologous recombination reinforces intraspecies cohesiveness [11]. Rates of homologous recombination vary widely within a given phylum, but are generally similar between species within a given genus [53]. Findings of homologous recombination events during bacterial evolution often lead to controversy regarding the concept of prokaryotic species and classification of prokaryotes [41,17]. Population genetics-based analyses have revealed association of interspecies recombination events in *Streptomyces* with historical demographic and biogeographic events [2].

Boetius et al. [3] reported high diversity of species, genetics, and metabolism in cold-adapted microorganisms that inhabit glacial environments. Such microorganisms have evolved over long periods of time under strong selection pressures such as low temperature and high UV radiation. There has been increasing description of novel bacterial taxa from glaciers during the past decade [56,13,26,58,59,27–32]. Dispersal limitation plays an important role in distribution of bacterial species on glaciers [33]; therefore, studies of such populations will help understand the relationships among evolution, homologous recombination, and historical biogeographic events.

The bacterial genus *Arthrobacter* is distributed worldwide in a variety of environments, including soil, water, glacier ice, and sewage. It is an excellent model system for evaluating the impact of gene exchange on diversification of bacterial taxa. All *Arthrobacter* species are Gram-positive actinomycetes with high G + C content [8]. *Arthrobacter* taxonomy has been the subject of longstanding controversy. On the basis of detailed analyses that included chemotaxonomic traits and phylogenetics, Busse proposed in 2016 [5] that five new genera be separated from *Arthrobacter*. *Arthrobacter*

* Corresponding author at: No. 1 West Beichen, Chaoyang District, Beijing 100101, China.
E-mail address: xinyh@im.ac.cn (Y.-H. Xin).

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is one of the most abundant bacterial genera in supraglacial habitats [34,47,40]. We have isolated numerous *Arthrobacter sensu lato* strains from glacier samples during the course of our studies on bacterial community structure, diversity patterns, and biogeography on glaciers in China [22] (Note: in following parts of this article, the term *Arthrobacter sensu lato* will signify *Arthrobacter* in the broad sense including Busse' new genera.).

Multiple housekeeping genes that evolve more quickly than 16S rRNA genes have been used for defining species within certain bacterial genera [43,37,7,49]. Multilocus sequences are also useful for analyzing patterns of genetic diversity and estimating the impact of homologous recombination on genetic differentiation and diversification in bacterial lineages [14,50,24]. In the present study, we conducted population genetics-based analyses of 41 type strains of *Arthrobacter sensu lato* using multilocus sequences, to elucidate the evolution and taxonomy of the genus. We also performed multilocus sequence analysis (MLSA) of 31 cold-adapted strains from four glaciers in China to infer differentiation among these populations separated by historical biogeographic barriers.

Materials and methods

Bacterial strains and culture conditions

Type strains of *Arthrobacter sensu lato*, including four of the new genera proposed by Busse, were obtained from China General Microbiological Culture Collection Center (CGMCC), Biological Resource Center, National Institute of Technology and Evaluation (NBRC), and Japan Collection of Microorganisms (JCM) (Supplementary Table S1). The strains were cultured under standard conditions for optimal growth as described in the online catalogue of CGMCC, NBRC and JCM. In addition, 31 *Arthrobacter sensu lato* strains (Supplementary Table S2) were selected for analysis from isolates we previously collected [33] from Xinjiang No. 1 glacier (NO1), Touming Mengke glacier (TM), Hailuogou glacier (HLG), and Midui glacier (MD). NO1 and TM are cold glaciers located in northwest China, whereas HLG and MD are temperate glaciers located in southwest China. The 31 glacier strains were incubated in PYG medium (Bacto Peptone (Difco) 0.5%, yeast extract 0.02%, glucose 0.5%, beef extract 0.3%, NaCl 0.05%, $MgSO_4 \cdot 7H_2O$ 0.15%, pH adjusted to 7.0) at 14 °C.

DNA extraction, amplification, and sequencing

Genomic DNA was extracted using a Genomic DNA Rapid Isolation Kit for Bacterial Cell (BioDev-Tech; Beijing, China) as per the manufacturer's instructions. Five single-copy housekeeping genes, *rpoB* (RNA polymerase β subunit), *recA* (homologous recombination factor), *atpD* (ATP synthase β subunit), *fusA* (elongation factor G), and *tuf* (translation elongation factor Tu), were amplified and sequenced using primers (listed in Table 1) that were designed using genomes of *Glutamicibacter arilaitensis* Re117^T (NC_014550), *Arthrobacter* sp. Rue61a (NC_018531), *Paenarthrobacter aurescens* TC1 (NC_008711), *Pseudarthrobacter chlorophenolicus* A6^T (NC_011886), *Arthrobacter crystallopoietes* BAB-32 (NZ_ANPE02000167), *A. flavus* TB 23 (NZ_ALPM01000021), *Paeniglutamicibacter gangotriensis* Lz1y^T (NZ_AOCK01000001.1), *Arthrobacter globiformis* NBRC 12137^T (NZ_BAEG01000078), *Arthrobacter* sp. FB24 (NC_008541), and *Pseudarthrobacter phenanthrenivorans* Sphe3^T (NC_015145). Primers for *secY* (protein translocase subunit) gene for present study were modified from Adekambi et al. [1]. PCR parameters for each gene: 94 °C, 5 min for initial denaturation; 30 cycles of 94 °C for 1 min, annealing temperature (Table 1) for 1 min, 72 °C for 1 min; final elongation at 72 °C for 10 min. Sequencing was performed using a PRISM 3730XL DNA analyzer (Applied Biosystems) at SinoGenoMax Co. (Beijing).

Table 1
PCR primers used to amplify *Arthrobacter sensu lato* genes *rpoB*, *secY*, *recA*, *atpD*, *tuf* and *fusA*.

Primer name	Primer sequence (5′–3′)	Amplicon size (bp)	Annealing temperature (°C)
rpoB2269f	GAAATCACYCGYGAYATCCC	850	58
rpoB3119r	CCRCCGAACTGTNCCTTACC		
secY232f[a]	GG**MA**TCATGCCSTACATYAC	826	54
secY1058r[a]	AA**B**CCRCC**GT**A**Y**TKCTTCAT		
recA373f	CARGCDYTGGARATCATGG	517	56
recA890r	TCDCCRTCRTASGTGAACC		
atpD568f	AACGACCTCTGGGTHGAAATG	668	56
atpD1236r	CTTNGCVGTRTAGGTGTTCT		
tuf374f	CCCGCCAGGTTGGYGTYC	756	60
tuf1130r	AAGCCGAGGCCYTCTTCC		
fusA391f	CCBCGYATCTGCTTCGTC	902	59
fusA1293r	SAGCTTYTCCTGGTCRCCCT		

[a] Primers for *secY* gene were modified from Adekambi et al. [1]; changes are shown in bold.

Nucleotide polymorphism

16S rRNA gene sequences of the above-mentioned strains were retrieved from GenBank using accession numbers. Sequences of 16S rRNA gene and six housekeeping genes were aligned using the MEGA 5.0 program [51] and trimmed manually. Sequences were concatenated using the SequenceMatrix program [54]. Locus characteristics, including number of segregating sites, nucleotide diversity (Pi), and Tajima's Neutrality Test, were determined by MEGA 5.0. Evolutionary distances were calculated using Kimura's two-parameter model [25].

Phylogenetic analysis and population structure

The GTR + G + I model was selected by jModelTest 2 [15] for construction of maximum likelihood (ML) trees. ML trees based on all loci and on concatenated sequences were created respectively by RAxML V. 8.1.1 [45] with 1000 bootstrap replicates, and MEGA 5.0 was used to build the neighbor-joining tree. Shimodaira–Hasegawa (SH) test [46] was used to evaluate incongruence between trees. Split phylogenetic networks for concatenated sequences were inferred using the SplitsTree4 program [22] with neighbor-net algorithm. Population structure was examined using the admixture model in the STRUCTURE software program [18]. Three independent STRUCTURE runs for each K of 2–10 were conducted with 200,000 burn-in iterations followed by 200,000 more. STRUCTURE Harvester [16] was used to choose the best ancestral population K. For the chosen K, the results of each run were compared and merged using the CLUMPP program [23]. Population differentiation (fixation index; Fst) was evaluated using DnaSP program V. 5.0 [35].

Assessment of recombination

Recombination was assessed by multiple methods. The SplitsTree4 program [22] was used to perform pairwise homoplasy index (PHI) test. Recombination events were detected using Recombination Detection Program (RDP) V. 4.80 [38] with default parameters (highest acceptable p-value 0.05). Recombination events were accepted if they were detected by three or more out of seven methods (RDP, Geneconv, Bootscan, MaxChi, Chimaera, SiScan and 3Seq) implemented in RDP. ClonalFrame [10] was used to infer the effect of recombination based on concatenated sequences with three independent runs. Following Markov Chain Monte Carlo (MCMC) steps consisting of 200,000 burn-in iterations plus 200,000

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