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Short Communication

Heavy metal resistant strains are widespread along *Streptomyces* phylogeny

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ABSTRACT

The genus *Streptomyces* comprises a group of bacteria species with high economic importance. Several of these species are employed at industrial scale for the production of useful compounds. Other characteristic found in different strains within this genus is their capability to tolerate high level of substances toxic for humans, heavy metals among them. Although several studies have been conducted in different species of the genus in order to disentangle the mechanisms associated to heavy metal resistance, little is known about how they have evolved along *Streptomyces* phylogeny. In this study we built the largest *Streptomyces* phylogeny generated up to date comprising six genes, 113 species of *Streptomyces* and 27 outgroups. The parsimony-based phylogenetic analysis indicated that (i) *Streptomyces* is monophyletic and (ii) it appears as sister clade of a group formed by *Kitasatospora* and *Streptacidiphilus* species, both genera also monophyletic. *Streptomyces* strains resistant to heavy metals are not confined to a single lineage but widespread along *Streptomyces* phylogeny. Our result in combination with genomic, physiological and biochemical data suggest that the resistance to heavy metals originated several times and by different mechanisms in *Streptomyces* history.

1. Introduction

The genus *Streptomyces* (Actinomycetales: Streptomycetaceae) comprises a group of bacteria species with high ecological and economic importance. In nature, species of *Streptomyces* play a major role in soil dynamic by degrading insoluble remains of other organisms and favoring carbon recycling (Zhou et al., 2012). From an economic point of view, these species are used at industrial scale for the production of different bioactive compounds: antibiotics, anticancer agents, herbicides, antiparasitic drugs and antifungals (Bentley et al., 2002; Hopwood, 2007). Due to the active secondary metabolism, streptomycetes also may be a good source for the identification of heavy metal binding components with possible future biotechnological application (Kothe et al., 2005). In fact, several strains of *Streptomyces* with resistance to different heavy metals have been isolated from contaminated areas (Amoroso et al., 1998, 2000, 2001; Polti et al., 2007; Albarracín et al., 2008; Haferburg et al., 2008; Siñeriz et al., 2009). Some *Streptomyces* species exhibit multiple tolerances against different metals and metaloids whereas others are more sensitive to them (Abbas and Edwards, 1989). The mechanisms associated to this resistance, nowadays focus of intensive research, include the production of extracellular quelators, an increased reductase activity, metal efflux pumps, intracellular sequestration and biomineralization (Schütze and Kothe, 2012).

Evolutionary studies, phylogenetic analysis among them, focused on organisms with economic importance are considered to give invaluable help in biological breeding. However, phylogenetic analyses on these organisms are seldom followed by studies that evaluate how useful properties have arisen and evolved. In the case of *Streptomyces*, nothing is known about the phylogenetic placement of the heavy metal resistant strains: do these all belong to the same clade or are these placed in different lineages?

Previously phylogenetic studies in *Streptomyces* included a reduced number of species (e.g. Guo et al., 2008; Alam et al., 2010; Rong and Huang, 2012), in general belonging to particular groups within the genus. Moreover, the outgroup sampling was very limited, in spite of the recognized importance of outgroup choice in phylogenetic analyses (Heath et al., 2008; Puslednik and Serb, 2008). For instance, Rong and Huang (2012) included only species of *Streptomyces* and Guo et al. (2008) included only *Micobacterium tuberculosis* as outgroup. In those cases where several outgroups were included, the sampling within *Streptomyces* was very limited

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(e.g. Alam et al., 2010). A phylogenetic analysis with wider taxon sampling is required to correctly analyze the distribution of heavy metal resistant strains. In this paper we analyze the distribution of heavy metal resistant strains along a newly generated phylogeny of *Streptomyces* that includes 113 species of the genus and 27 outgroups. Our results are analyzed in conjunction with genomic, biochemical and physiological data in order to better comprehend the origin and evolution of heavy metal resistance in *Streptomyces*.

2. Methods

2.1. Phylogenetic reconstruction

The phylogenetic analysis included all the species of *Streptomyces* that have sequences in GenBank for the following six genes: 16S rRNA, *atp*D, *gyr*B, *trp*B, *rpo*B and *rec*A. The dataset included the sequences generated for the analyses of Guo et al. (2008) and Rong and Huang (2012) and plus all the species of *Streptomyces* whose genomes were sequenced at June 2012. For each species, the sequences of the six genes belonged to the same strain. The analysis included 113 species of *Streptomyces* (Supplementary Table 1). In addition, a total of 27 outgroups were also included: nine species of *Kitasatospora*, eight species of *Streptacidiphilus* (both of Streptomycetaceae family), nine species from other families of Actinomycetales and *Bifidobacterium longum* (Bifidobacteriales). The final matrix comprised 140 species and 10,030 characters. The alignments were run in Mafft (Katoh et al., 2005) using the G-INS-i algorithm. The alignments were manually edited to exclude regions with ambiguous alignment. Gaps were treated as missing.

The phylogenetic searches were conducted using parsimony and Maximum Likelihood (ML) criteria. Parsimony analyses were run in TNT (Goloboff et al., 2008). Each run started from a Random Addition Sequence (RAS) followed by TBR. After that, the trees were submitted to a combination of Sectorial Searches (SS), Tree Drifting (TD), Ratchet and Tree Fusing (Goloboff, 1999). Multiple runs were conducted and the analysis was stopped when the minimum length was independently obtained 30 times. Clade support was assessed by absolute jackknifing frequencies with a removal probability of 0.36 (Goloboff et al., 2003). The search strategy in each jackknifing replicate comprised 30 RAS followed by TBR + SS and TD. The ML analysis was conducted in RAxML (Stamatakis, 2006). The search involved ten independent parsimony trees as starting points followed by ML search with the GTRMIX option (i.e. using the GTRCAT approach during runs and calculating the final ML scores using GTRGAMMA model).

The dataset was built with the aid of GenBank-to-TNT (Goloboff and Catalano, in press). This is a pipeline for easily creating molecular matrices, starting from GenBank files and finishing with phylogenetic matrices that can be read by TNT program (Goloboff et al., 2008). GB-to-TNT is designed to retrieve a defined genomic region from a bulk of sequences included in a GenBank format file.

2.2. Phylogenetic placement of strains resistant to heavy metals

The first step in the analysis was to record from bibliography all the strains cited to be resistant to heavy metals and that had 16S rRNA sequences available (Supplementary Table 2). We assume as valid the definition of resistant strain given in each publication, including in Supplementary Table 2 all the information about levels of resistance and experimental conditions of each resistant strain. Once retrieved from GenBank, the 16S rRNA sequences belonging to the resistant strains were aligned together with the 16S dataset used in the six-gene *Streptomyces* phylogenetic analysis previously described. The distribution of resistant strains along *Streptomyces* phylogeny was evaluated in three different ways. (i) Calculating the optimal position (in terms of parsimony score) of each resistant strain on the species level phylogeny of *Streptomyces* inferred in this work (i.e. using the six-gene species level phylogeny as backbone), (ii) performing a phylogenetic search considering only the 16s dataset and (iii) performing a combined phylogenetic analysis that included all the sequences from the six-gene phylogeny plus those of the heavy metal resistant strains. All the analyses were conducted using scripts written by SAC (available from the authors upon request).

3. Results and discussion

The present analysis represents the most comprehensive phylogenetic reconstruction generated up to date for the genus *Streptomyces*. The strict consensus of the parsimony analysis is shown in Fig. 1. The consensus is well resolved with many clades presenting high support values. The general structure of the Maximum Likelihood tree is in agreement with that of the parsimony analysis (Supplementary Fig. 1). Streptomycetaceae appears as monophyletic, presenting two main clades, one of them including all the species of *Streptacidiphilus* and *Katenulispora*, both genera monophyletic, and a second clade corresponding to *Streptomyces*. The resolution within *Streptomyces* is high in general, with several clades having moderate to high support. Except of the case of *S. griseus*, strains of the same species were not grouped according to their specific status. In fact, the monophyly was strongly rejected when performing constrained searches: forcing the monophyly of each species produced scores that were clearly suboptimal. The case of *S. hygroscopicus* is paradigmatic in this respect since the shortest tree with this species as monophyletic was 474 steps longer than the most parsimonious trees. Although less considerable, the monophyly of other species was also clearly contradicted: *S. rimosus* (+seven steps), *S. libani* (+64 steps). This result is in agreement with those previously obtained considering smaller taxon samplings (Guo et al., 2008; Rong and Huang, 2012).

The analysis of the distribution of heavy metal resistant strains on *Streptomyces* phylogeny clearly showed that the resistant strains are not confined to a single lineage within *Streptomyces*, but that are widespread along its phylogeny (Fig. 1; Supplementary Figs. 2 and 3). An exception of this pattern is the lack of resistant strains in a large clade comprising 25 terminals[1]. This clade presents a high support value and was also retrieved by Rong and Huang (2012). A potential limitation of our analysis is related to the possible lack of enough phylogenetic information in the 16S sequences included in the analysis that may preclude to discern the position of the resistant strains on *Streptomyces* phylogeny. A thorough analysis of our results allows us to discard this possibility. When analyzing the optimal position of resistant strain on the six-gene phylogeny, the strains that presented only 16s sequences were optimally placed in either a unique branch of the tree or in neighbor branches, hence indicating that there was indeed enough phylogenetic information in 16s sequences. A similar result was obtained when the placement of resistant strains was evaluated by performing a phylogenetic search that included these strains. The lack of phylogenetic information in 16s would have produced a strict consensus completely unresolved, and this was not the case for our analysis (Fig. 3 Supplementary Data). And more important, the resis-

[1] If a binomial distribution is used in a simplified way to statistically test this pattern, the probability that no resistant strains fall into this clade by chance would be 2×10^{-5}. This probability is artificially low because the strains isolated from the same locality cannot be considered as independent. However, if a conservative position is taken and the number of resistant strains is equated to the number of localities the probability is still significant ($P < 0.01$).

Fig. 1. Strict consensus of the four optimal trees (26,823 steps) obtained in the parsimony analysis. Arrows indicate the position of the heavy metal resistant strains. The frame indicates a clade comprising 35 terminals with no resistant strain (see text).

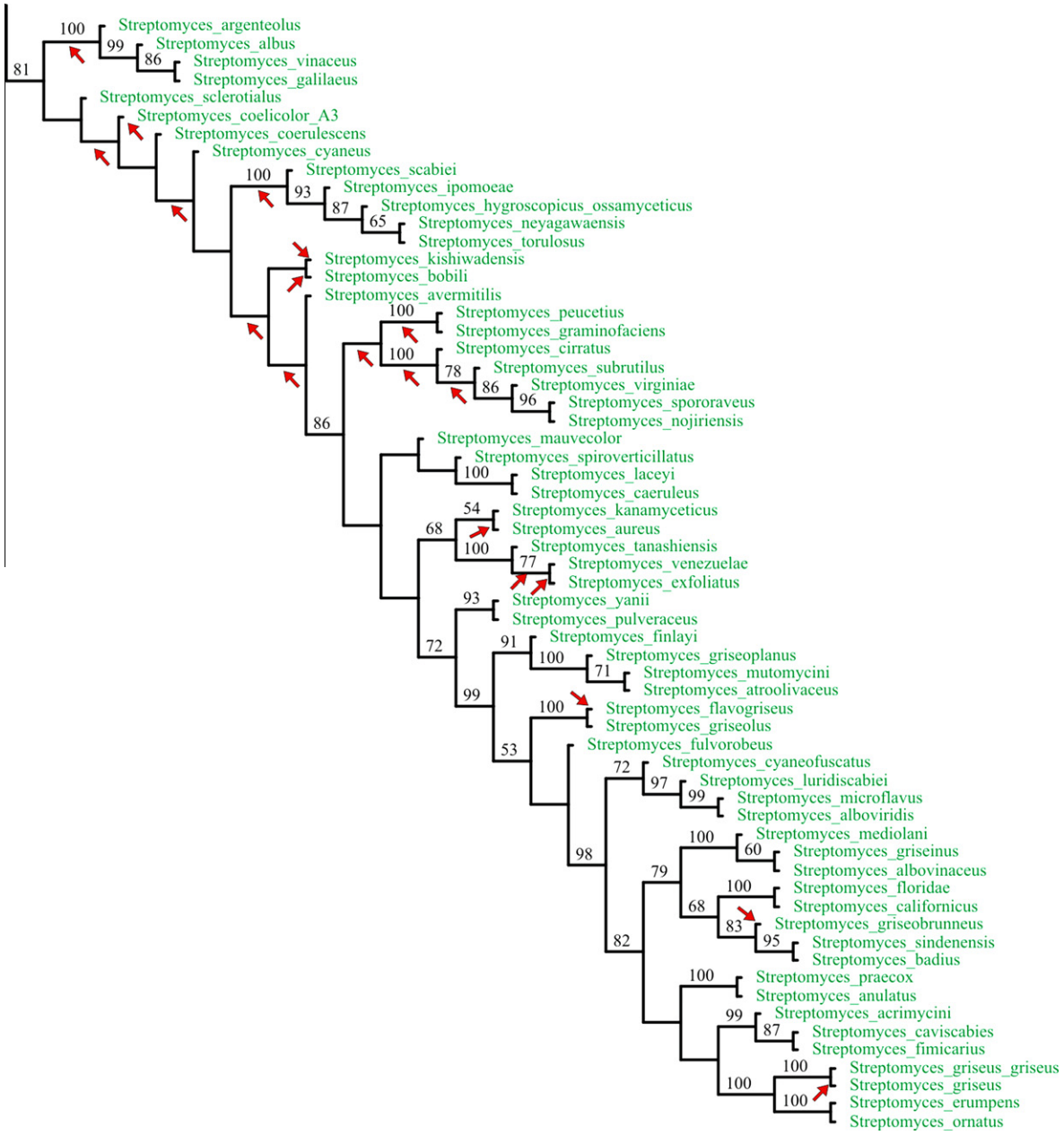

Fig. 1. (*continued*)

tant strains appeared again in different clades of the strict consensus, also supporting the widespread distribution of resistant strains.

Different analyses (e.g. Doroghazi and Buckley, 2010; Laskaris et al., 2010) have indicated that horizontal gene transfer (HGT) in Streptomyces is not only associated to adaptation genes but also to housekeeping genes. Consequently, HTG can potentially affect the results of the present phylogenetic analysis. The effect of HTG on the phylogenetic results can be recognized by the lack of resolution in the resulting consensus and/or lower clade support (but see Bapteste et al., 2008). Having obtained high support values for many clades in our analysis suggest that, although HTG may have been an important process in the evolution of *Streptomyces*, it did not blurred out the phylogenetic signal. In addition, and most important for the goals of this study, many of the heavy metal resistant strains were placed in clades that are strongly supported, indicating the conclusions about the evolution of the resistance along *Streptomyces* phylogeny holds in spite of the potential negative effect of HTG in the phylogenetic reconstruction.

The presence of heavy metal resistant strains in different *Streptomyces* clades may have two different explanations: (i) the resistance was already present in the most recent common ancestor (MRCA) and was then inherited by the different lineages (ii) the different lineages inherited from the MRCA the capacity to develop new mechanisms, or modify existing ones, in order generate resistance to heavy metals. Although a complete evaluation of these competing hypotheses would require having disentangled the genetic and biochemical mechanisms that confer the resistance in each strain, a comprehensive assessment of the existing information about this issue allows evaluating the available evidence that support each hypothesis.

Schütze and Kothe (2012) indicated that several morphological, physiological and reproductive characteristics of *Streptomyces* (the filamentous growth, the formation of hyphae and the production of spores) would allow its species to occupy extreme environments. Since these properties are shared by all species of the genus, this can be interpreted as evidence that supports the inheritance of

resistance to heavy metals from the MRCA. This is in agreement with Zhou et al. (2012) who indicated that the impressive machinery for biosynthesis that present *Streptomyces* species inherited from their MRCA would be an adaptive trait for surviving in adverse environment. Physiological and biochemical studies in different *Streptomyces* strains have shown that resistance to heavy metals is produced by several mechanisms, acting alone or in conjunction: an increased reductase activity; production of extracellular quelators; metal efflux pumps; intracellular sequestration and biomineralization (Schütze and Kothe, 2012). This contrast with the idea of a single origin for the metal tolerance and suggest that it was not the tolerance itself but the capability to developed new mechanisms that has been inherited from the MRCA of *Streptomyces*. Genomic data can give invaluable help to understand the causes behind the presence of resistant strains in different lineages of *Streptomyces*. According to Bentley et al. (2002) the linear chromosome of *Streptomyces* was essential for the occupation of a variety of environments, contaminated environments among then, by species of this genus. The genome of *Streptomyces* presents about 8–9 mb probably derived from a four mb actinomycete ancestor (Hopwood, 2006). The "core genome" carries most of the housekeeping genes and occupies the central region of the chromosome. In contrast the "arms" of the *Streptomyces* genome carries mainly conditionally adaptive genes and were probably generated by gene duplication and lateral transfer (Hopwood, 2006). The comparison of the genomes of *S. avermitilis* and *S. coelicolor* indicated that they contain a different set of gene clusters for secondary metabolism suggesting that the arm regions of different streptomycete chromosomes have been accumulated separately, containing different complement of contingency genes (Bentley et al., 2002). These large differences among the genomes of *Streptomyces* species can also be seen in the number of copies of some gene families: in some cases reaching a 10-fold difference (Zhou et al., 2012). Obtaining new genes by gene duplication and lateral transfer has probably been facilitated the linear chromosome that allowed the expansion of the genome without substantially altering the fitness of the organism (Volff and Altenbuchner, 1998). Consequently, the linear genome would be a key innovation responsible for the multiple derivation of heavy metal resistance in *Streptomyces*. There is at least one documented case where a large insertion that included genes related to metal resistance (Bentley et al., 2002).

Genes associated to heavy metal resistance are not only confined to the chromosome in *Streptomyces* but also to plasmids (Ravel et al., 1998). There is evidence that the resistance can be acquire by the lateral transfer of this plasmids between different strains of *Streptomyces* (Ravel et al., 2000). Hence, besides chromosome structure and the metabolic machinery, the widespread presence of resistant strains in *Streptomyces* can be related to the transfer of plasmids with genes that confer resistance.

In conclusion, the evidence obtained in our analysis combined with genomic, biochemical and physiological information suggest a scenery where the acquisition of resistance to heavy metals in *Streptomyces* is a consequence of a dynamic process – probably helped by a linear genome and an impressive metabolic machinery – that involve the independent development of the resistance in several lineages and/or lateral transfer. However, further experimental studies should be conducted in order to better comprehend the origin and evolution of heavy metal tolerance in *Streptomyces*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.11.025.

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