Taxonomic analysis of *Sorangium* strains based on HSP60 and 16S rRNA gene sequences and morphology

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The taxonomy of myxobacteria is based mainly on their morphological characteristics. The genus *Sorangium* belongs to the myxobacterial suborder *Sorangiiineae*. Strains in the genus were classified either as one species, *Sorangium cellulosum*, by ignoring divergent morphological characteristics, or into several species; however, the latter classification is based on some dubious morphological characteristics and is inconsistent with the phylogeny constructed from 16S rRNA gene sequences. In this study, two HSP60 (*groEL1* and *groEL2*) genes were amplified and sequenced from 22 *Sorangium* strains. The *groEL1* and *groEL2* gene sequences were highly conserved in *Sorangium* strains, suggesting that these two paralogous genes both play important roles in the life cycle. The phylogeny constructed by the *groEL* genes was rather consistent with the morphological characteristics of sporangioles. Including information from the phylogenetic analysis and morphological characteristics, it is suggested that the genus *Sorangium* includes two species.

Classical taxonomy of myxobacteria was established based mainly on morphological characteristics (McCurdy, 1989; Reichenbach & Dworkin, 1992). While stable morphological characteristics such as shapes of vegetative cells and myxospores are consistent with the classification of suborders, the genera or species of myxobacteria have generally been divided on the basis of some dubious morphological characteristics such as the structure and colour of fruiting bodies (Reichenbach, 2005; Shimkets *et al.*, 2006).

The genus *Sorangium* is located in the suborder *Sorangiiineae*. Its members are remarkable among the 19 myxobacterial genera not only for their unique ability to degrade cellulosic materials (Reichenbach, 2005; Hou *et al.*, 2006) but also for their excellent capacity to produce various bioactive metabolites (Gerth *et al.*, 2003). *Sorangium* strains have been classified either into one determinate species, *Sorangium cellulosum*, suggested to contain many variants, or into several species (Reichenbach, 2005), or were tentatively divided into three

species based mainly on the colour of fruiting bodies, whereby strains with orange, dark brown and nearly black fruiting bodies were named '*Sorangium compositum*', *S. cellulosum* and '*Sorangium nigrum*', respectively (Shimkets *et al.*, 2006). However, the names '*S. compositum*' and '*S. nigrum*' have not been validly published (<http://www.bacterio.cict.fr/s/sorangium.html>). Although the present morphology-based taxonomy of the genus *Sorangium* is coherent, species are often inconsistent with the phylogenetic results from 16S rRNA gene sequences. The 60 kDa heat-shock protein (HSP60; also known as GroEL) belongs to the chaperonin family (Lin & Rye, 2006). HSP60 is ubiquitous and highly conserved in bacteria, and thus is being used more frequently to infer eubacterial phylogenies (Karlin & Brocchieri, 2000). The *groEL* gene is present as a single copy in most bacterial species, but two or more copies are found in some bacteria (Goyal *et al.*, 2006). In this paper, two different HSP60 genes (*groEL1* and *groEL2*) were amplified from *Sorangium* strains. Phylogenetic analyses based on HSP60 and 16S rRNA gene sequences were compared comprehensively with the morphological taxonomy for 22 *Sorangium* strains.

Morphologies of the *Sorangium* strains

The morphological properties of some of the 22 *Sorangium* strains used in this study are listed in Table 1. The strains were routinely cultivated on CNST medium, containing (w/v) 0.05 % KNO₃, 0.025 % Na₂HPO₄ · 12H₂O, 0.1 %

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are EU240489–EU240498 and EU240530–EU240533 (16S rRNA genes), EU240499–EU240520 (*groEL1*) and EU240521–EU240529 (*groEL2*).

Micrographs of *Sorangium* strains and an alignment of 16S rRNA gene sequences are available as supplementary material with the online version of this paper.

Table 1. Major morphological characteristics of *Sorangium* strains

Morphological characteristics of the nine other strains included in this study, So9987-5, So9735-22, So9881, So9721-1, So9711-1, So9733-1, So0089-1, So9741 and So ce26, were reported previously (Yan *et al.*, 2003). Strains DSM 14627^T and DSM 14731 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen. Strain So ce26 was provided by Hans Reichenbach. The other strains shown, isolated and maintained by the authors, were chosen from hundreds to represent strains with diverse morphological characteristics. NA, Not applicable.

Strain	Size (µm)		Sporangiole		Fruiting body		
	Vegetative cells	Myxospores	Shape	Size (µm)	Colour	Texture	Distribution
DSM 14627 ^T	6–8 × 0.8–1	2–3 × 0.8–1	Spherical, in parcels	15–20	Yellow	Soft	Whole
So0087-23	4–8 × 0.8–1	2–3 × 0.8–1	Spherical, in parcels	10–15	Red–orange	Rigid	Whole
So0085-1	4–7 × 0.8–1	2–4 × 0.8–1	Spherical, in parcels	10–15	Red–orange	Rigid	Rusted region
So0157-1	6–8 × 0.8–1	2–3 × 0.8–1	Spherical, in parcels	10–15	Red–orange	Soft	Whole
So0157-24	4–7 × 0.8–1	2–3 × 0.8–1	Spherical, in parcels	20–30	Yellow	Soft	Rusted region
So0157-52	4–6 × 0.8–1	1–2 × 0.8–1	Spherical, unpacked	5–10	NA	NA	NA
So0133-4	5–6 × 0.8–1	1–2 × 0.8–1	Spherical, in parcels	10–15	Yellow	Soft	Whole
So0081-1	4–6 × 0.8–1	2–3 × 0.8–1	Spherical, in parcels	15–20	Yellow	Rigid	Whole
So9734-1	4–7 × 0.8–1	2–3 × 0.8–1	Spherical, in parcels	10–15	Red–orange	Soft	Whole
So0139-5	4–6 × 0.8–1	2–3 × 0.8–1	Spherical, unpacked	15–25	NA	NA	NA
So0184-1	4–7 × 0.8–1	2–3 × 0.8–1	Spherical, in parcels	10–20	Yellow	Soft	Whole
So0007-16	4–7 × 0.8–1	2–3 × 0.8–1	None	NA	NA	NA	NA
DSM 14731	6–8 × 0.8–1	2–3 × 0.8–1	Polyhedral, in parcels	15–20	Black	Soft	Whole

MgSO₄·7H₂O, 0.001 % FeCl₃·6H₂O, 1.5 % agar and 1 ml trace elements solution l⁻¹ (Yan *et al.*, 2003). Morphologies were determined using the methods described in a previous report (Yan *et al.*, 2003).

The sporangioles, which contain many embedded myxospores, were either spherical or polyhedral in shape and varied in size. They were often packed into macroscopic fruiting bodies, but unpacked sporangioles were also found in some strains, such as So0157-52 and So9721-1. Most of the 22 strains had yellow to red–orange fruiting bodies, while some strains, i.e. So0089-1, So ce26, So9741 and DSM 14731, had a deeper colour of brown or black. Fruiting mostly appeared with the whole swarm on partially or completely degraded filter paper, while some strains formed fruiting bodies only in the swarm centre (So9741), at the swarm edge (So9881) or at undegraded places far beyond the degraded regions (So ce26). Although the characteristics of the fruiting bodies have been one of the important taxonomic criteria for the classification of species in *Sorangium*, this characteristic was not stable and either degenerated or was lost in many strains after rounds of cultivation. Some strains, such as So9987-5, So0007-16 and So0139-5, had no sporangiole structures when they were initially isolated. Members of the *Sorangineae* have been shown to be able to etch agar (Reichenbach & Dworkin, 1992), but the ability varied greatly among different strains. On filter paper-CNST medium, the extension of sorangial swarms also varied among the strains. All of the 22 strains had the ability to degrade cellulose, and a piece of filter paper with an area of 1 cm² was degraded completely after 7–14 days of cultivation at 30 °C. Supplementary Fig. S1, available in IJSEM Online,

demonstrates the typical morphologies, especially the sporangioles, of the *Sorangium* strains.

Phylogenetic analysis based on 16S rRNA gene sequences

Extraction of genomic DNA from cells and PCR amplification and sequencing of 16S rRNA genes were performed using previously described methods (Li *et al.*, 2002). For phylogenetic analysis, nucleotide sequences were aligned using the CLUSTAL_X computer program (version 1.83; Thompson *et al.*, 1997). The aligned sequences were refined and then analysed phylogenetically by distance/neighbour-joining programs with the Poisson correction distance model in the MEGA3.1 software package (Kumar *et al.*, 2004) to infer their phylogenetic relationships. The phylogenetic relationships of the sequences were also confirmed by the likelihood and parsimony programs in the PHYLIP software package (v. 3.61) (Felsenstein, 2001). Bootstrapping support for the interior branch lengths of the tree was obtained from 1000 replicates. Reference sequences were extracted from GenBank.

Fig. 1(a) shows the phylogenetic tree constructed from the 16S rRNA gene sequences of all the *Sorangium* strains examined. The phylogenetic tree was highly consistent with that constructed with 10 *Sorangium* strains by Yan *et al.* (2003). The sequence similarity of the *Sorangium* strains to the cellulolytic species *Byssovorax cruenta* was 94–96 %. Within *Sorangium*, the divergence was less than 2.3 %, except strain So9741, which exhibited the lowest sequence interstrain similarity, of 96.8 %, in comparison with So ce26. The topology of the tree separated the strains into

Fig. 1. Phylogenetic analysis based on 16S rRNA gene sequences (a) and HSP60 gene sequences (b) of 22 *Sorangium* strains and related reference myxobacteria strains. Aligned sequences of the two types of HSP60 gene (*groEL1* and *groEL2*) were extracted to the same length of about 600 bp. In (b), branches mentioned in the text are indicated by the roman numerals I–V, and groups of spherical and polyhedral sporangiole-forming strains are indicated. Bars, 2 changes per 100 nucleotide positions. Numbers on branch nodes indicate bootstrap support percentages based on 1000 replicates.

four groups. However, this separation was not supported by morphological characteristics.

To analyse the nucleotide changes in the 16S rRNA gene sequences of *Sorangium* strains in detail, the sequences were aligned corresponding to the conserved and variable regions of bacterial 16S rRNA gene sequences, and divergent nucleotides were highlighted (Supplementary Fig. S2). There were 96 sites in the sequence that contained one or two divergent nucleotides, including many unique inserts. The divergent sites were scattered along the 16S rRNA gene sequence, not only in the variable regions but also in conserved regions. Compared with the other 21 strains, So9741 had many unique bases in conserved regions C3, C4, C5 and C6. This clearly indicated that strain So9741 should be separated from the other strains as a member of a different species. Although So ce26 formed a separate clade from the others in the phylogenetic tree, the strain did not contain many unique bases. The divergent sites of So ce26 appeared mainly in the variable regions V2 and V4. It is also interesting to see that, in So9987-5, there were nine unique two-base inserts in the 16S rRNA gene sequence, but the phylogenetic tree did not reflect this change. Obviously, gaps had been removed before phylogenetic analysis and were not included. The results revealed that the phylogeny constructed from the *Sorangium* 16S rRNA gene sequences provides little information for the classification of species.

Phylogenetic analysis based on HSP60 sequences

Screening the published myxobacterial genomes revealed two copies of the HSP60 gene (*groEL1* and *groEL2*). The degenerate primers H279 and H280 described by Goh *et al.* (1996) were first used for the amplification of the conserved segment of *groEL1* (with an expected length of about 600 bp) from the *Sorangium* strains. The 25 µl PCR mixture consisted of approximately 300 ng DNA template, 2.5 nmol each dNTP, 2.5 U *Taq* DNA polymerase, 1 × GC buffer and 50 pmol each of primers H279 and H280. The thermal cycling conditions were as follows: 96 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 46 °C for 1 min and 72 °C for 1 min. Finally, the reaction was maintained at 72 °C for 10 min. This degenerate primer set produced

positive results from 14 of the 22 *Sorangium* strains. A new *Sorangium*-specific primer set, HSP60MyxoF and HSP60MyxoR, was designed based on the sequences of the *groEL1* genes that were amplified from *Sorangium* strains by using the H279/H280 primer set. Our new myxobacteria-specific primers were able to amplify the gene segment from all of the *Sorangium* strains that we studied using the above PCR conditions with the slight modification of setting the annealing temperature to 70 °C. Two new degenerate primers, HSP60Myxo2F and HSP60Myxo2R, were also designed on the basis of the sequences of putative *groEL2* proteins from the three published myxobacteria genomes, those of *Myxococcus xanthus* DK1622 (GenBank accession no. CP000113), *Anaeromyxobacter dehalogenans* 2CP-C (CP000251) and *Anaeromyxobacter* sp. Fw109-5 (CP000769). The PCR amplification was modified by adding 100 pmol of each of the primers to the reaction mixture and setting the annealing temperature to 57 °C and the extension time to 2.5 min. The primers produced positive results from nine of the 22 *Sorangium* strains (expected products of about 1440 bp). The products were analysed by electrophoresis in 1 % (w/v) agarose gel in 1 × TAE buffer (containing 50 mM Tris/acetate, 1 mM EDTA, pH 8.0), stained with ethidium bromide and visualized under UV light. After purification using an agarose gel DNA extraction kit (Roche), the amplified DNA sequences were ligated into the pGEM-T Easy vector (Promega) following the protocols recommended by the manufacturer. The ligation mixtures were transferred by electroporation into *Escherichia coli* JM109 cells using the standard method (Sambrook & Russell, 2001). Sequencing was performed at the Sangon Sequencing Center (Shanghai, China). The primers employed for amplification of the *groEL1* and *groEL2* gene sequences are listed in Table 2.

The sequence similarity between the 22 *Sorangium groEL1* genes was 94.2–99.6 %. The nine *groEL2* sequences were also highly similar, with more than 96 % identity. Phylogenetic trees reconstructed from the complete and partial sequences of the *groEL2* gene were nearly the same (data not shown), and thus the same segments of *groEL1* and *groEL2* were combined to reconstruct a phylogenetic tree (Fig. 1b). The two *groEL* genes were grouped separately. The sequences within each *groEL* gene showed

Table 2. Primers for PCR amplification of *groEL* genes from *Sorangium* strains

Inosine (I) was used to reduce the degeneracy of the primers. Primers were synthesized at Sangon Sequencing Center (Shanghai, China).

Primer	Sequence (5'–3')	Target	Reference
H279	GAATTCGAIHHGICGGIGA(TC)GGIACIACI	<i>groEL1</i>	Goh <i>et al.</i> (1996)
H280	CGCGGGATCC(TC)(TG)I(TC)(TG)ITCICC(AG)AAICCGGIGC(TC)TT	<i>groEL1</i>	Goh <i>et al.</i> (1996)
HSP60MyxoF	ACGACGACGGCGAC(GC)GTGCT(GC)GC	<i>groEL1</i>	This study
HSP60MyxoR	TCGCC(AG)AAGCCGGGGGC(CT)TT(GC)AC	<i>groEL1</i>	This study
HSP60Myxo2F	GT(GC)AC(GC)CT(GC)GG(GC)CC(GC)AAGGG	<i>groEL2</i>	This study
HSP60Myxo2R	CT(GC)GC(GC)GCGTTCTG(GC)AG(GC)GC	<i>groEL2</i>	This study

high levels of similarity, which probably indicates that both genes are important, but play different roles in *Sorangium* cells. The *groEL1* genes were divided into five branches, and phylogenetic analysis based on the nine *groEL2* gene sequences was consistent with the tree of *groEL1* gene sequences, with only small exceptions within the branches. The similarity between *groEL1* and *groEL2* in the same *Sorangium* strain was highly stable, ranging between 71.5 and 73.3%. The sequence similarity between the *Sorangium groEL1* sequences and the reference myxobacterial *groEL1* sequences varied from 70.6–74.4% (*Anaeromyxobacter* strains) to 67.8–70.6% (*M. xanthus* DK1622), while the similarity between the *Sorangium groEL2* sequences and the reference myxobacterial *groEL2* sequences was 73.6–77.7% (*Anaeromyxobacter* strains) to 70.1–73.1% (*M. xanthus* DK1622). In contrast, the sequence similarity of the two *groEL* genes was 86.2 and 82.5% in the two *Anaeromyxobacter* strains and 83.8% in *M. xanthus* DK1622. These results suggest that *groEL1* and *groEL2* are paralogous genes in myxobacteria. To date, there have been no reports on the functions of the two *groEL* genes in myxobacteria, although they have been reported in two-dimensional proteome screens (Dahl *et al.*, 2007; Otani *et al.*, 2001). It will be very interesting to discover their functions in the life cycle.

In the *groEL1* tree, strain So ce26 was also separated from others, and strain So9741 had the lowest sequence similarity to other strains (94.2–95%) with the exception of its fellow *groEL1* group member DSM 14731 (97.9%). However, the two trees reconstructed from the *groEL* and 16S rRNA genes were not highly congruent. Comparison of the *groEL1* phylogeny and morphological characteristics revealed some significant results. The *groEL1* branches were consistent with the shapes of sporangioles, but not with any other morphological characteristics. For example, the sporangioles of the members of branch I were spherical and packed into macroscopic fruiting bodies, while their *groEL1* sequences showed similarity of more than 97.2%. Branch IV was composed of two strains, DSM 14731 and So9741, whose sporangioles were polyhedral and were packed to form fruiting bodies. *groEL1* sequences divided the *Sorangium* strains with spherical sporangioles into three subgroups (branches I, II and III) and the strains with polyhedral sporangioles into two subgroups (branches IV and V). The results were consistent with the conclusion of our previous paper (Yan *et al.*, 2003), i.e. rather than the colour of the fruiting bodies, sporangiole shape is more important as a morphological taxonomic criterion to separate species in the genus *Sorangium*. The colour of the fruiting bodies was also consistent with the *groEL1*-based classification, but there were some exceptions. For instance, strain So0089-1 had black and spherical sporangioles, but was located on branch III.

Sorangium strains have gigantic genomes, more than 13 Mb in *S. cellulosum* strain So ce56 (Schneiker *et al.*, 2007). Bioinformatic analysis of the genome reveals that a large percentage of the genome is devoted to regulation, which

probably supports its complex social lifestyle and thus diversified morphologies. It has long been suggested that there should be more than one species within the genus, and there have been attempts to divide *Sorangium* strains based on their morphological characteristics (Reichenbach & Dworkin, 1992; Reichenbach, 2005; Shimkets *et al.*, 2006). However, phylogenetic analysis of 16S rRNA gene sequences did not separate the *Sorangium* strains clearly (Yan *et al.*, 2003). More significantly, the morphological taxonomy of *Sorangium* is often incompatible with the phylogeny, due to the high similarity of the 16S rRNA gene sequences of *Sorangium* strains. Interestingly, the two HSP60 genes produced highly similar phylogenetic results, and can be divided into several obvious groups. In the classical taxonomy of the *Myxococcales*, morphology is the primary basis for classification. The types of fruiting bodies and sporangioles form the basis of classification of genera, and sizes of myxospores or other fine morphological differences have been used to define each species. The phylogenetic tree constructed from the *groEL* genes is consistent with the morphological characteristics of sporangioles. As mentioned previously, spherical sporangioles have often been observed to coexist with polyhedral sporangioles, which were the variants of the spherical sporangioles, clearly formed by dense packing (Reichenbach, 2005). On the other hand, many *Sorangium* strains contain only spherical sporangioles, whether they were densely or loosely packed. In the case of strains So ce26, So9741 and DSM 14731, the polyhedral sporangiole shape is a rather stable characteristic even when the strains were cultivated on different media. Additionally, polyhedral sporangioles were usually found mixed with many stuffing substrates, forming fruiting bodies (Yan *et al.*, 2003). The stuffing materials were not able to be observed in fruiting bodies containing spherical or mixed-shaped sporangioles. Thus, the *Sorangium* strains could be divided, at least, into two groups, containing spherical or polyhedral shapes of sporangioles. For final classification of species or subspecies in *Sorangium*, more phenotypic as well as genotypic analyses are needed.

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