Phytoactinopolyspora endophytica gen. nov., sp. nov., a halotolerant filamentous actinomycete isolated from the roots of Glycyrrhiza uralensis F.

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A novel endophytic actinomycete, designated strain EGI 60009T, was isolated from the roots of Glycyrrhiza uralensis F. collected from Xinjiang Province, north-west China. The isolate was able to grow in the presence of 0–9 % (w/v) NaCl. Strain EGI 60009T had particular morphological properties: the substrate mycelia fragmented into rod-like elements and aerial mycelia differentiated into short spore chains. LL-2, 6-Diaminopimelic acid was the cell-wall diamino acid and rhamnose, galactose and glucose were the cell-wall sugars. MK-9(H4) was the predominant menaquinone. The major fatty acids of strain EGI 60009T were iso-C15 : 0, anteiso-C15 : 0, anteiso-C17 : 0, iso-C17 : 0, iso-C17 : 1 and I/anteiso-C17 : 0 B. Mycolic acids were absent. The DNA G + C content of strain EGI 60009T was 70.4 mol%. Phylogenetic analysis based on the 16S rRNA gene sequence indicated that strain EGI 60009T belongs to the family Jiangellaceae and formed a distinct clade in the phylogenetic tree. 16S rRNA gene sequence similarities between strain EGI 60009T and other members of the genera Jiangella and Haloactinopolyspora were 96.1–96.4 and 95.7–96.0 %, respectively. Based on these results and supported by morphological, physiological and chemotaxonomic data and numerous phenotypic differences, a novel species of a new genus, Phytoactinopolyspora endophytica gen. nov., sp. nov., is proposed. The type strain of Phytoactinopolyspora endophytica is EGI 60009T (=KCTC 29657T=CPCC204078T).

The family Jiangellaceae was first described by Tang et al. (2011). It comprises, at the time of writing, two genera with validly published names: Jiangella (Song et al., 2005) and Haloactinopolyspora (Tang et al., 2011; Zhang et al., 2014), the two genera having distinctive morphological and/or chemotaxonomic characteristics. The genus Jiangella comprises four recognized species isolated from desert soil, cave, medicinal plant and mouldy cell wall, respectively (Song et al., 2005; Lee, 2008; Qin et al., 2009; Kämpfer et al., 2011). The genus Haloactinopolyspora includes two species, Haloactinopolyspora alba (Tang et al., 2011) and Haloactinopolyspora alkaliphila (Zhang et al., 2014), isolated from a salt lake and a saline–alkali soil, respectively.

Strain EGI 60009T was isolated from the healthy roots of Glycyrrhiza uralensis F. collected from Yili County, Xinjiang Province, north-west China.
chemotaxonomic properties of strain EGI 60009\(^T\) indicated that it belongs to the family Jiangellaceae. Phylogenetic analysis based on 16S rRNA gene sequence data showed that strain EGI 60009\(^T\) formed a separate lineage within the family Jiangellaceae. Therefore, we propose that strain EGI 60009\(^T\) should be classified as representing a novel species of a new genus in the family Jiangellaceae.

Glycyrrhiza uralensis F. samples were washed in running tap water to remove soil particles and were surface-sterilized first with 95 % ethanol for 5 min, then with 0.1 % (w/v) mercuric chloride for 4 min and rinsed five times with sterile water according to Vincent (1970). Then, the surface-sterilized root samples were sliced into small pieces, followed by plating on R2A agar (BD) containing nalidixic acid (25 mg l\(^{-1}\)) and nystatin (100 mg l\(^{-1}\)) to repress growth of bacteria and fungi. Plates were incubated at 28 °C for 4 weeks until the outgrowth of endophytic actinomycetes was discerned. A single colony was selected and purified by re-cultivation onto R2A plates and incubated again. One isolate, EGI 60009\(^T\), was cultured on R2A agar at 28 °C and maintained on R2A agar slants at 4 °C and as 20 % (v/v) glycerol suspensions at −80 °C.

Cultural characteristics of strain EGI 60009\(^T\) were recorded on International Streptomyces Project (ISP) media (Shirling & Gottlieb, 1966), Czapek’s agar, nutrient agar, triptiase soy agar (TSA) and R2A agar. All media were supplemented with 2 % (w/v) NaCl for growth. The colours of substrate and aerial mycelia and any soluble pigments produced were determined by comparison with chips from ISCC-NBS colour charts (Kelly, 1964). Growth was good on nutrient agar, R2A agar, Czapek’s agar, inorganic salts/starch agar (ISP 4) and oatmeal agar (ISP 3); there was poor growth on TSA and no growth on yeast extract/malt extract agar (ISP 2) or glycerol/asparagine agar (ISP 5). The aerial mycelium colour was white and that of the substrate mycelium was white–yellow. No soluble pigments were produced in all the media tested. Morphological characteristics of strain EGI 60009\(^T\) were observed by light microscopy (model BH 2; Olympus) and scanning electron microscopy (JSM5600LV; JEOL) after cultivation on nutrient agar containing 2 % (w/v) NaCl at 28 °C for 2 weeks. The substrate mycelia were well developed and fragmented into rod-like elements, while the aerial mycelia had short spore chains (Fig. 1).

Growth at different temperatures (4, 10, 20, 28, 37, 40, 42, 45, 50 and 55 °C) was tested on nutrient agar plates containing 2 % (w/v) NaCl for 2 weeks. For NaCl tolerance experiments, nutrient agar medium was used as the basal medium, and salt concentrations ranging from 0 to 15 % (w/v), at intervals of 1 %, were tested at 28 °C for 2 weeks. The pH growth range was investigated between pH 4.0 and 10.0, at intervals of 1 pH unit, using the buffer system described by Tang et al. (2011). Anaerobic growth was determined using the GasPak Anaerobic System (BBL), according to the manufacturer’s instructions. Catalase, oxidase and gelatinase activities, starch hydrolysis, nitrate reduction, H\(_2\)S production and urease were assessed as described by Smibert & Krieg (1994). Carbon source utilization was determined as described by Williams et al. (1989). Enzyme activities were determined by using the API ZYM system (bioMérieux), according to the manufacturer’s instructions. Antibiotic sensitivity was explored by placing commercial antibiotic discs (HiMedia) on the modified nutrient agar plates that had been spread with the isolates and then incubated at 28 °C for 5 days.

Fig. 1. Scanning electron micrographs of spore chains of strain EGI 60009\(^T\) grown on nutrient agar with 2 % (w/v) NaCl for 1–2 weeks at 28 °C. (a) Substrate mycelium fragmented into rod-like elements; aerial mycelium was present when cultured for 7 days. (b) Short spore chains after culture for 2 weeks. Bars, 5 μm (a), 10 μm (b).
Strain EGI 60009<sup>T</sup> was able to grow at 20–37 °C, at pH 6.0–9.0 and with 0–9 % NaCl. The isolate was sensitive to tetracycline (30 μg), gentamicin (10 μg), vancomycin (30 μg), penicillin (10 IU), chloramphenicol (30 μg), novobiocin (5 μg), tobramycin (10 μg), erythromycin (15 μg), rifampicin (5 μg), ampicillin (10 μg) and amikacin (30 μg), but resistant to norfloxacin (10 μg) and ciprofloxacin (5 μg). The detailed physiological and biochemical characteristics of the isolate are given in the species description.

The isomer of diaminopimelic acid was analysed according to the procedure developed by Hasegawa et al. (1983). Amino acids in cell-wall hydrolysates were analysed by precolumn derivatization with o-phthalaldehyde by HPLC (Tang et al., 2009a). Cell-wall sugars were detected by precolumn derivatization with 1-phenyl-3-methyl-5-pyrazolone by HPLC (Tang et al., 2009b). Polar lipids were extracted as described by Minnikin et al. (1984) and identified by two-dimensional TLC (Collins & Jones, 1980). Menaquinones were extracted (Collins et al., 1977) and analysed by HPLC (Tamaoka et al., 1983). Mycolic acids were extracted and analysed according to the protocol of Minnikin et al. (1984). For fatty acid analysis, cells of strain EGI 60009<sup>T</sup> and the reference strains were analysed on nutrient agar (BD) containing 2 % NaCl at 28 °C for 7 days. Analysis of the cellular fatty acid pattern followed the method described by Sasser (1990), but we used version 6.0 of the Sherlock Microbial Identification System (MIDI). The genomic DNA was prepared according to the method of Marmur (1961). The G+C content of the DNA was determined by reversed-phase HPLC of nucleosides according to Mesbah et al. (1989).

The results indicated that strain EGI 60009<sup>T</sup> contained LL-2, 6-diaminopimelic acid (LL-DAP), alanine, aspartate and glutamic acid as cell-wall amino acids. Glucose, galactose, mannose and rhamnose were the major cell-wall sugars. The polar lipids were diphasophatidylglycerol, phosphatidylglycerol, phosphatidylinositol mannosides, three unknown phosphoglycolipids and four unknown phospholipids (Fig. S1, available in the online Supplementary Material). The predominant menaquinone was MK-9(H<sub>4</sub>) (99.2 %), with a minor amount of MK-9(H<sub>6</sub>) (0.8 %) detected. Strain EGI 60009<sup>T</sup> had a cellular fatty acid profile that contained major amounts of branched fatty acids and minor amounts of saturated and unsaturated fatty acids. The major fatty acids were anteiso-C<sub>15</sub>:0 (23.1 %), anteiso-C<sub>17</sub>:0 (16.9 %), iso-C<sub>17</sub>:0 (11.4 %), iso-C<sub>15</sub>:0 (11.1 %) and iso-C<sub>17</sub>:1ω9c and anteiso-C<sub>17</sub>:0 (11.1 %); a moderate amount of iso-C<sub>16</sub>:0 (8.0 %) was found, and the minor fatty acids were C<sub>14</sub>:0 (1.2 %), C<sub>16</sub>:0 (2.7 %), C<sub>17</sub>:0 (1.1 %), C<sub>13</sub>:0 (1.1 %), iso-C<sub>16</sub>:1G (0.8 %), anteiso-C<sub>17</sub>:0 (0.8 %), C<sub>17</sub>:0 (1.5 %), C<sub>18</sub>:0 (1.0 %), iso-C<sub>14</sub>:0 (0.9 %), iso-C<sub>16</sub>:0 (0.9 %), C<sub>15</sub>:0 (2.4 %), iso-C<sub>16</sub>:0 (3.0 %), C<sub>17</sub>:0 (2.8 %) and C<sub>16</sub>:1ω7c/C<sub>16</sub>:1ω6c (2.2 %). Mycolic acids were absent (Fig. S2). The G+C content of the DNA was 70.4 mol%.

The extraction of genomic DNA and PCR amplification of the 16S rRNA gene were done as described by Li et al. (2007). Multiple alignments with sequences of the suborder Propionibacterineae and calculations of levels of sequence similarity were carried out using EzBio Cloud (http://www.eztaxon.org; Kim et al., 2012). Phylogenetic analyses were performed using three tree-making algorithms: the neighbour-joining (Saitou & Nei, 1987), maximum-likelihood (Felsenstein, 1981) and maximum-parsimony (Fitch, 1971) methods. A phylogenetic tree was reconstructed using the neighbour-joining method of Saitou & Nei (1987) from K<sub>me</sub> values (Kimura, 1980) using MEGA version 6.0 Tamura et al. (2013). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

The almost-complete 16S rRNA gene sequence of strain EGI 60009<sup>T</sup> comprised 1514 bp. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the organism clustered with members of the genera *Jiangella* and *Haloactinopolyspora* (Fig. 2). Levels of 16S rRNA gene sequence similarity between strain EGI 60009<sup>T</sup> and the type strains of *Jiangella alba*, *Jiangella gansuensis*, *Jiangella muralis*, *Jiangella alkaliphila*, *H. alba* and *H. alkaliphila* were 96.4, 96.2, 96.1, 96.0 and 95.7 %, respectively. In the phylogenetic tree based on the neighbour-joining algorithm, strain EGI 60009<sup>T</sup> and members of the genera *Jiangella* and *Haloactinopolyspora* clustered in a distinct clade supported by a high bootstrap value (100 %). This relationship was supported by all the tree-making methods used in this study. Although strain EGI 60009<sup>T</sup> was similar to members of the genera *Jiangella* and *Haloactinopolyspora* with MK-9(H<sub>4</sub>) as the predominant menaquinone and LL-DAP as the cell-wall diamino acid, it differed based on morphological properties (Fig. 1). Moreover, strain EGI 60009<sup>T</sup> could be distinguished from members of the genus *Jiangella* in having short spore chains. In addition, strain EGI 60009<sup>T</sup> exhibited chemotaxonomic differences from the genus *Jiangella*. Strain EGI 60009<sup>T</sup> contained glucose, galactose, mannose and rhamnose (no ribose) as major cell-wall sugars, anteiso-C<sub>15</sub>:0, anteiso-C<sub>17</sub>:0, iso-C<sub>15</sub>:0, iso-C<sub>17</sub>:1ω9c and iso-C<sub>17</sub>:0ω9c as major fatty acids, and diphasophatidylglycerol, phosphatidylglycerol, three unknown phosphoglycolipids, four unknown phospholipids and phosphatidylinositol mannosides as polar lipids (Fig. S1). Members of the genus *Jiangella* contain glucose and ribose as cell-wall sugars (rhamnose, mannose or galactose may be present), anteiso-C<sub>15</sub>:0, iso-C<sub>16</sub>:0 and iso-C<sub>17</sub>:1ω9c as major fatty acids (iso-C<sub>14</sub>:0 or iso-C<sub>15</sub>:0 or iso-C<sub>16</sub>:0 may also be present as major fatty acids), and phosphatidylinositol as the predominant polar lipid (phosphatidylcholine may also be present) (Table 1; see also Table S1 and Fig. S1). The isolated strain could also be distinguished from members of the genus *Haloactinopolyspora* (Table 1). The latter contain glucosamine, glucose, galactose, mannose and arabinose as cell-wall sugars, anteiso-C<sub>15</sub>:0, iso-C<sub>16</sub>:0 and anteiso-C<sub>17</sub>:0 as major fatty acids, and phosphatidylinositol and
an unknown glycolipid as the predominant polar lipids (Tang et al., 2011; Zhang et al., 2014). In particular, strain EGI 60009\textsuperscript{T} was morphologically distinct from members of the genus Haloactinopolyspora (Fig. 1): strain EGI 60009\textsuperscript{T} did not form pseudosporangium-like, rhiziform spore aggregates at maturity.

The 16S rRNA gene sequences of strain EGI 60009\textsuperscript{T} and all members of the family Jiangellaceae were scanned for signature nucleotides; strain EGI 60009\textsuperscript{T} and the type strains of members of the two related genera Jiangella and Haloactinospora had many unique 16S rRNA gene signature nucleotides, but also showed two specific positions at 127 : 234 (G–C) and 833 : 853 (G–C). Therefore, on the basis of differences in phenotypic, chemotaxonomic and phylogenetic characteristics between the new isolate and its closest neighbours, the genera Jiangella, Couchioplanes caeruleus subsp. caeruleus NBRC 13939\textsuperscript{T} (D85479)

Pseudosporangium ferrugineum 3-4-4-a-19\textsuperscript{T} (AB302183)

Krasinkovia cinnamomea 3-54/41\textsuperscript{T} (AB236956)

Xiangella phaseoli NEAU-J5\textsuperscript{*} (JQ073792)

Henricospora githakensis DSM 44339\textsuperscript{*} (Y15523)

Jishengella endophytica 202201\textsuperscript*T (EU560726)

Plantactinospora siamensis CM2-8\textsuperscript{*} (AB454379)

Salinispora arenicola CNH-643\textsuperscript{T} (AY404619)

Polymorphopshora rubra TT 97-42\textsuperscript{T} (AB223089)

Actinoplanes philippinensis ATCC 12427\textsuperscript{T} (U58525)

Polymorphospora rubra TT 97-42\textsuperscript{T} (AB223089)

Asanoa ferruginea IMSU 22009\textsuperscript{T} (AF152108)

Catenuloplanes japonicus DSM 44102\textsuperscript{T} (X93201)

Luedemannella helvata 3-9/24\textsuperscript{T} (AB236957)

Allocatellobiospora scoiae Sco-B14\textsuperscript{T} (FN386736)

Catellatospora citrea subsp. citrea IMSU 22008\textsuperscript{T} (AF152106)

Phytomonospora endophytica gen. nov., sp. nov. EGI 60009\textsuperscript{T} (KP271925)

Pseudonocardia seranimata YIM 63233\textsuperscript{T} (FJ817379)

Pseudonocardia sichuanensis KLBMP 1115\textsuperscript{T} (HM153789)

Pseudonocardia saturnea IMSU 20052\textsuperscript{T} (AJ252829)

Pseudonocardia alaniniphila Y-16303\textsuperscript{T} (EU722519)

Pseudonocardia salamisensis K236\textsuperscript{T} (JQ864427)

Actinoalloteichus nanshanensis NEAU 119\textsuperscript{T} (GQ926935)

Saccharopolyspora erythraea NRRL 2338\textsuperscript{T} (AM420293)

Goodfellowiella coeruleoviolacea NRRL B-20458\textsuperscript{T} (DQ093349)

Streptoalloteichus hindustanus IFO 15115\textsuperscript{T} (D85497)

Kutzneria albida DSM 43870\textsuperscript{T} (CP007155)

Allokutzneria albata DSM 44149\textsuperscript{T} (AJ512462)

Lechevalieria atacamensis C61\textsuperscript{T} (EU551684)

Lechevalieria roselyniae C81\textsuperscript{T} (EU551683)

Lechevalieria deserti C68\textsuperscript{T} (EU551682)

Stackebrandtia nassauensis NRRL B-16338\textsuperscript{T} (AY650268)

Halorygoscomycetes albus YIM 92370\textsuperscript{T} (EU660053)

Glycomyces harbinensis IFO 14487\textsuperscript{T} (D85483)

Bifidobacterium gallicum JCM 8224\textsuperscript{T} (D86189)

Fig. 2. Phylogenetic relationships between strain EGI 60009\textsuperscript{T} and members of the family Jiangellaceae and the type strains of related species on the All-Species Living Tree based on the 16S rRNA gene sequences. The branching pattern was generated by the neighbour-joining method. Bootstrap values (expressed as percentages of 1000 replications) above 50% are shown at branch points. Bar, 0.02 substitutions per nucleotide position.
Table 1. Differential phenotypic characteristics between strain EGI 6009\(^{\dagger}\) and related members of the genera *Jiangella* and *Haloactinopolyspora*

| Strains: 1, EGI 6009\(^{\dagger}\); 2, *J. gansuensis* YIM 002\(^{\dagger}\); 3, *J. alkaliphila* DSM 45079\(^{\dagger}\); 4, *J. muralis* YIM 61503\(^{\dagger}\); 5, *J. alba* DSM 45357\(^{\dagger}\); 6, *H. alkaliphila* EGI 80088\(^{\dagger}\) (Zhang et al., 2014); 7, *H. alba* YIM 93246\(^{T}\) (Tang et al., 2011). All data for members of the genus *Jiangella* are from the present study except aerial mycelium characteristics, cell-wall sugars, polar lipids and genomic DNA G+C contents for *J. gansuensis* YIM 002\(^{\dagger}\), *J. alkaliphila* DSM 45079\(^{\dagger}\), *J. alba* YIM 61503\(^{\dagger}\) and *J. muralis* DSM 45357\(^{\dagger}\), which are from Song et al. (2005), Lee (2008), Qin et al. (2009) and Kämpfer et al. (2011), respectively. The reference type strains were cultivated at their optimum culture conditions. +, Positive/utilized; +\(\dagger\), weakly positive; –, negative/not utilized; ND, not detected; Ara, arabinose; Gal, galactose; GlcN, glucosamine; Glu, glucose; Man, mannose; Rha, rhamnose; Rib, ribose; DPG, diphosphatidylglycerol; PC, phosphatidylcholine; PG, phosphatidylglycerol; GL, unknown glycolipid; PGL, unknown phosphoglycolipid; PI, phosphatidylinositol; PIM, phosphatidylinositol mannoside; PL, unknown phospholipid; UL, unknown polar lipid.

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Phytoactinopolyspora endophytica gen. nov., sp. nov.
and Haloactinopolyspora, we suggest that strain EGI 60009\(^T\) represents a novel species of a new genus, for which the name Phytoactinopolyspora endophytica gen. nov., sp. nov. is proposed.

**Description of Phytoactinopolyspora gen. nov.**

*Phytoactinopolyspora* (Phy.to.ac.ti.no.po.ly.spo’ra. Gr. n. phyt\(\omicron\)on plant; Gr. n. actis actinos a ray; Gr. adj. poly many; Gr. n. spora a seed and, in biology, a spore; N.L. fem. n. Phytoactinopolyspora many-spored ray isolated from plant tissues).

Gram-reaction-positive, strictly aerobic, moderately halotolerant filamentous actinomycetes. The substrate mycelium fragments into rod-like elements, and the aerial mycelium has long or short spore chains. The cell-wall hydrolysates contain LL-DAP, alanine, aspartate and glutamic acid as the cell-wall amino acids; glucose, galactose, mannose and rhamnose are major cell-wall sugars. The polar lipids are diposphatidylglycerol, phosphatidylinositol, phosphatidylinositol mannosides, three unknown phosphoglycolipids and four unknown phospholipids. The predominant menaquinone is MK-9 (H\(4\)). The major fatty acids are anteiso-C\(_{15}:0\), anteiso-C\(_{17}:0\), iso-C\(_{17}:0\), iso-C\(_{15}:0\) and iso-C\(_{17}:1\) anteiso-C\(_{17}:0\). Mycolic acids are absent. The G\(+\)C content of the DNA is about 70 mol%. The type species is *Phytoactinopolyspora endophytica*.

**Description of Phytoactinopolyspora endophytica sp. nov.**

*Phytoactinopolyspora endophytica* (en.do.phy’ti.ca. Gr. pref. endo- within; Gr. n. phyt\(\omicron\)on plant; Gr. n. suff. -ica adjectival suffix used with the sense of belonging to; N.L. fem. adj. endophytica within plant, endophytic).

In addition to the morphological, chemotaxonomic and general characteristics described for the genus, colonies of the isolate develop well on ISP 3, ISP 4, Czapek’s agar, R2A agar and nutrient agar media. Poor growth occurs on TSA and no growth occurs on ISP 2 and ISP 5 agar. Soluble pigment is not produced on tested media. The colour of colonies is white on the tested media. Growth occurs at 20–37 \(^\circ\)C and pH 6.0–9.0. Tolerates up to 9 % (w/v) NaCl. Optimal temperature and pH for growth are 28 \(^\circ\)C and pH 7.0. Positive for catalase, oxidase, gelatin liquefaction, nitrate reduction and hydrolysis of Tweens 40, 60 and 80. Negative for urease, milk coagulation, milk peptonization, H\(_2\)S production, and hydrolysis of Tween 20, cellulose and starch. D-Mannitol, D-galactose, L-arabinose, z-lactose, melibiose, raffinose, melezitose, D- rhamnose, D-xyllose, D-mannose, D-glucose, sucrose and glycerol can be used as sole carbon sources for growth, but not D-sorbitol, maltose, cellobiose, turanose, L-sorbose, inositol, D-fructose, malate or citrate. L-Alanine, L-asparagine, L-tyrosine, hypoxanthine, xanthine and adenine can be used as sole nitrogen sources, but not glutamine, threonine, aspartic acid or glycine. In the API ZYM system, alkaline phosphatase, acid phosphatase,
esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase and α-fucosidase are positive; α-galactosidase, β-glucuronidase and N-acetyl-β-glucosaminidase are negative.

The type strain, EGI 60009T (=KCTC 29657T=CPCC 204078T), was isolated from surface-sterilized roots of Glycyrrhiza uralensis F. collected from Yili County, Xinjiang Province, north-west China. The DNA G+C content of the type strain is 70.4 mol%.

Acknowledgements

This work was supported by National Natural Science Foundation of China (No. 31200008) and the West Light Foundation of the Chinese Academy of Sciences, China National Program of High Technology Research and Development (Grant nos. 2012AA021705 and 2012AA063503), W. H. and W.-J. L. extend their appreciation to the Deanship of Scientific Research at King Saud University for funding this work through the research group no. RGP-205.W.-J.L. was also supported by Hundred Talents Program of the Chinese Academy of Sciences and Guangdong Province Higher Vocationa...