



## 基于基因组的一株土壤固氮菌分离菌株鉴定及其促生作用

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**摘要:** 【目的】为获得高效固氮菌株, 充分研究利用土壤固氮菌资源。【方法】选取固氮能力较高的紫色土发育水稻土, 采用富集纯化法分离固氮微生物菌株。通过16S rRNA基因系统发育分析和全基因组相关指数比较对新分离菌株进行物种鉴定。采用乙炔还原法和<sup>15</sup>N<sub>2</sub>示踪法定量测定新分离菌株的固氮能力, 通过培养特性和接种效果初步研究固氮菌株的促生作用。【结果】从紫色土发育水稻土中分离得到1株可在无氮培养基上快速生长的菌株P208。基于16S rRNA基因和基因组92个核心基因的系统发育分析结果表明, 新分离菌株P208与*Azotobacter chroococcum* IAM 12666<sup>T</sup>(=ATCC 9043<sup>T</sup>)系统发育距离最近(16S rRNA基因相似度为99.79%)。菌株P208与*A. chroococcum* ATCC 9043<sup>T</sup>的基因组平均核苷酸一致性(ANI)、平均氨基酸一致性(AAI)和数字DNA-DNA杂交值(dDDH)高于物种分类阈值(ANI>95%–96%, AAI>95%–96%, dDDH>70%), 最大唯一匹配指数(MUMi)低于物种分类阈值(<0.33), 得出新分离菌株P208为褐球固氮菌(*A. chroococcum*)。 *A. chroococcum* P208固氮活性为模式菌株*A. chroococcum* ATCC 9043<sup>T</sup>的2.61倍。除固氮能力外, *A. chroococcum* P208具有IAA生成、溶磷活性和铁载体生成等促进植物生长潜力的培养特性, 室内培养条件下接种*A. chroococcum* P208能够促进水稻、小麦幼苗根系的生长。【结论】从固氮能力较强的水稻土中分离纯化得到1株具有较强固氮、促生潜力的固氮菌, 具有潜在的开发应用价值, 可为研究利用生物固氮提供微生物资源。

**关键词:** 生物固氮, 固氮菌, 褐球固氮菌, 促生作用, 促生菌

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生物固氮(Biological nitrogen fixation, BNF)是固氮微生物将氮气转化为生物可利用活性氮的过程,作为氮循环的重要组成部分,是氮素可利用转化的主要自然途径<sup>[1-4]</sup>。与化学氮肥相比,固氮微生物固定的氮素是农业生态系统中环境友好的活性氮来源。最大化地开发利用生物固氮作用,从而减少化学氮肥的施用量,有利于农业的可持续发展。开展固氮菌资源的收集及特性研究对于实现固氮菌资源有效利用、提高农田生物固氮能力具有重要的现实意义。

非共生固氮(asymbiotic nitrogen fixation)是不依靠微生物与植物形成共生关系的生物固氮形式,在陆地生态系统中广泛分布<sup>[3]</sup>。除固氮作用外,前人研究中分离获得的非共生固氮菌株大多可在体外检测到生长素的生成,部分菌株还具有铁载体生成和溶磷活性等促进植物生长潜力<sup>[5-6]</sup>。前人研究表明,接种固氮促生菌能够对甘蔗、水稻、玉米、小麦等作物的生长产生多方面有益作用<sup>[7-10]</sup>。在巴西、阿根廷等南美洲地区,商业化的固氮促生菌(固氮螺菌 *Azospirillum* spp.)接种剂应用较为普遍,约有 350 万  $\text{hm}^2$  的小麦、玉米和高粱等作物已被接种,且使用量还在不断增加<sup>[11-12]</sup>。在阿根廷, *Azospirillum brasilense* Az39 用于玉米、小麦和其他非豆类作物的接种在全国范围内推广,在研究和应用推广中,增产效果得到了充分证实<sup>[13-14]</sup>。相比之下,国内的非共生固氮微生物接种剂产品较少且推广应用效果不够理想。

微生物菌株的分离培养工作是微生物学研究的基础,分离获得微生物菌株是微生物系统分类学和微生物资源利用的重要基础<sup>[15-16]</sup>,纯培养物挖掘越来越受到国内外微生物研究学者的重

视。近年来,国内研究学者从农田土壤、作物体内分离筛选得到了越来越多的非共生固氮菌种资源<sup>[17-19]</sup>。固氮酶基因的定量及无氮培养基培养计数的结果均表明,土壤中的固氮菌显著多于根系<sup>[20]</sup>。水稻土的淹水环境为固氮微生物提供了良好的生存环境<sup>[21-22]</sup>,是固氮菌株分离的重要来源。课题组前期研究<sup>[23]</sup>表明,不同类型水稻土的生物固氮量差异很大,紫色土发育水稻土的固氮能力显著高于其他水稻土类型(红壤发育水稻土、黑土发育水稻土和下位砂姜土发育水稻土)。因此,本研究选用固氮能力较高的紫色土发育水稻土作为分离来源,通过富集纯化法分离固氮微生物菌株,采用基于基因组的方法对其进行物种水平鉴定,并对其固氮能力和促生作用进行研究,以评价其开发应用潜力,为研究利用生物固氮提供微生物资源。

## 1 材料和方法

### 1.1 土壤样品

本研究分离土壤固氮菌的来源为紫色土发育水稻土,采自四川省盐亭县水稻田耕层土壤(31°16'14"N, 105°28'27"E),根据课题组前期研究结果<sup>[23]</sup>,紫色土发育水稻土的固氮能力较高,约为红壤发育水稻土的 9.14 倍,约为黑土发育水稻土的 3.00 倍,约为下位砂姜土发育水稻土的 2.14 倍。

### 1.2 培养基

Brown 无氮培养基<sup>[24]</sup>(g/L): 葡萄糖 5, 无水  $\text{CaCl}_2$  0.15,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.2,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.04,  $\text{Na}_2\text{MoO}_4$  0.005,  $\text{K}_2\text{HPO}_4$  0.8, pH 6.8-7.0。固体培养基另加琼脂糖 10-15 g/L。

### 1.3 菌株的分离

采用富集纯化法分离高效固氮菌株。取 10 g 土壤样品加入 90 mL 液体无氮培养基中, 28 °C 振荡培养 4–5 d (180 r/min), 取 1% 的培养物接种到 100 mL 液体无氮培养基中进一步培养, 之后每 2–3 d 转接 1 次。重复转接 4 次后, 在固体无氮培养基上采用稀释平板法分离, 挑取单菌落划线纯化。

### 1.4 菌株基因组 DNA 提取

挑取纯化的单菌落接种于液体无氮培养基中, 28 °C、180 r/min 振荡培养至  $OD_{600} \approx 1$ , 吸取 1 mL 菌液于 1.5 mL 离心管, 离心收集菌体 (10000×g, 10 min), 用细菌基因组 DNA 提取试剂盒 (TaKaRa, Code No. 9763) 提取菌株基因组 DNA, ddH<sub>2</sub>O 溶解洗脱 DNA。

### 1.5 菌株 16S rRNA 基因序列分析

采用细菌通用引物 27F (5'-AGAGTTTGATC MTGGCTCAG-3') 和 1492R (5'-GGTTACCTTGTT ACGACTT-3')<sup>[25–26]</sup> 对 16S rRNA 基因全长序列进行 PCR 扩增。PCR 反应中的 DNA Polymerase、Buffer、dNTPs Mixture 等使用 TaKaRa Premix Taq (Ex Taq Version 2.0 plus dye) (TaKaRa, Code No. RR902A), 其中包含: TaKaRa Ex Taq 0.05 U/μL、dNTPs Mixture 0.4 mmol/L、Mg<sup>2+</sup> 4 mmol/L。PCR 反应体系包含: 25 μL TaKaRa Premix Taq、50–100 ng 基因组 DNA、1 μL 正向引物 (20 μmol/L)、1 μL 反向引物 (20 μmol/L)、ddH<sub>2</sub>O 补充至 50 μL。PCR 反应条件为: 94 °C 5 min; 94 °C 1 min, 55 °C 1 min, 72 °C 1.5 min, 30 个循环; 72 °C 5 min。

PCR 扩增后, 以 DL2000 (TaKaRa, Code No. 3427Q) 为 DNA Marker, 对扩增产物进行 1% 琼脂

糖凝胶电泳。目的 DNA 片段回收纯化后, 通过 3730×1 DNA Analyzer (Applied Biosystems) 进行测序, 测序结果使用 SeqMan (DNASTAR Lasergene) 拼接。使用 EzBioCloud Identify service (<https://www.ezbiocloud.net/identify>)<sup>[27]</sup> 将所得序列与数据库进行比对。

从数据库中收集相似度较高或相近科属的模式菌株 16S rRNA 基因序列以重建系统发育树, 在 MEGA 7 中采用 Neighbor-Joining 方法进行系统发育树构建<sup>[28–29]</sup>。Neighbor-Joining 建树模式为 Tamura 3-parameter model, 使用 Bootstrap 进行检验评估, 设置复制值为 1000 次。

### 1.6 菌株全基因组测序与相关指数比较

使用 NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs) 进行文库构建。利用 Illumina HiSeq 测序平台进行 2×150 bp 测序, 对测序数据通过 FastQC 进行质量评估, 通过 Trimmomatic 0.36 进行质量剪切<sup>[30]</sup>。使用 SPAdes 3.5.0 进行基因组拼接<sup>[31]</sup>, 采用 NCBI prokaryotic genome annotation pipeline (PGAP) 进行基因组注释<sup>[32]</sup>。

从公共数据库中收集了相近模式菌株的基因组序列, 使用 Up-to-date bacterial core gene set and pipeline (UBCG) 重建基于基因组 92 个核心基因的系统发育树<sup>[33]</sup>, 通过基因支持指数 (gene support index, GSI) 评估系统发育分支。

测序获得新分离菌株 P208 的全基因组序列 (GCA\_005144545.1), 从公共数据库收集密切相关的模式菌株 *Azotobacter chroococcum* ATCC 9043<sup>T</sup> (GCA\_004327905.1) 和 *A. beijerinckii* DSM 378<sup>T</sup> (GCA\_900110885.1) 基因组序列作为对比, 计算菌株之间的全基因组相关指数<sup>[34]</sup>。使用

JSpeciesWS Online Service (<http://jspecies.ribohost.com/jspeciesws/>)计算平均核苷酸一致性(average nucleotide identity, ANI)和四核苷酸频率相关系数(tetranucleotide frequency correlation coefficient, TETRA)<sup>[35]</sup>。使用 Genome-to-Genome Distance Calculator 2.1 (<http://ggdc.dsmz.de/ggdc.php>)计算数字 DNA-DNA 杂交值(digital DNA-DNA hybridization, dDDH)与 G+C 含量差异<sup>[36]</sup>。利用在线工具(<http://enve-omics.ce.gatech.edu/aai/>)计算平均氨基酸一致性(average amino acid identity, AAI)<sup>[37]</sup>。利用 INRA-MUMi 在线工具(<http://genome.jouy.inra.fr/mumi/>)计算最大唯一匹配指数(maximal unique matches index, MUMi)<sup>[38]</sup>。

### 1.7 菌株固氮能力的定量测定

采用乙炔还原法和  $^{15}\text{N}_2$  示踪法测定对数生长中期( $OD_{600}\approx 0.4-0.8$ )菌液的固氮能力, 菌液蛋白量采用 Bradford 法测定<sup>[39]</sup>, 牛血清蛋白(BSA)作为蛋白标准。选择固氮菌属模式菌株 *A. chroococcum* ATCC 9043<sup>T</sup> 作为对照。

对于乙炔还原法, 将对数生长中期菌液转移到无菌玻璃瓶中。置换顶空 10% 的空气为高纯乙炔。黑暗条件下 28 °C、180 r/min 振摇培养 2 h, 采集瓶内顶空气体, 气相色谱法测定顶空乙烯产生情况<sup>[40]</sup>, 同时做无菌培养基对照和无乙炔置换对照。

对于  $^{15}\text{N}_2$  示踪法, 将对数生长中期菌液转移到无菌玻璃瓶中。置换顶空 10% 的空气为高纯  $^{15}\text{N}_2$ 。黑暗条件下 28 °C、180 r/min 振摇培养 2 h, 通过 Whatman 玻璃微纤维过滤器(GF/F) (GE Healthcare, CAT No.1825-047)过滤收集细胞, 烘干称重研磨后采用元素分析仪-同位素比质谱仪(EA-IRMS) (Flash 2000-Delta V advantage, Thermo

Fisher Scientific)测定氮含量与  $^{15}\text{N}$  丰度<sup>[41]</sup>。

### 1.8 菌株促生潜力体外测定

对新分离菌株的 IAA 生成、溶磷活性和铁载体生成进行定量分析, 并与固氮菌属模式菌株 *A. chroococcum* ATCC 9043<sup>T</sup> 进行对比。

**1.8.1 IAA 生成测定:** 菌株振摇培养于含有 500 mg/L L-色氨酸的液体 Brown 培养基中(28 °C、180 r/min), 48 h 后取菌液离心(10000×g, 10 min), 取上清液 1 mL, 加入显色液(10.8 mol/L  $\text{H}_2\text{SO}_4$ , 4.5 g/L  $\text{FeCl}_3$ ) 1 mL 混合均匀, 室温避光反应 30 min 后测定其  $OD_{540}$ 。同时以吲哚-3-乙酸标准液绘制标准曲线<sup>[42]</sup>。

**1.8.2 溶磷活性测定:** 菌株振摇培养于溶磷 NBRIP 培养基<sup>[43]</sup>中(28 °C、180 r/min), 7 d 后取菌液离心(10000×g, 10 min), 取上清液用钼蓝比色法测定菌液中的可溶性磷含量<sup>[44-45]</sup>。

**1.8.3 铁载体生成测定:** 菌株振摇培养于无铁的液体 Brown 培养基中(28 °C、180 r/min), 72 h 后取菌液离心(10000×g, 10 min), 取上清液 1 mL, 加入 CAS 检测液<sup>[46]</sup>1 mL 混合均匀, 室温反应 1 h 后测定  $OD_{630}$ , 以未接菌培养基作为参比对照。

### 1.9 菌液接种对作物生长的影响

接种液准备: 将无氮培养基中 28 °C、180 r/min 振摇培养 24 h 的菌液离心(5000×g, 5 min)、无菌水漂洗后重悬, 调节菌浓度约为  $10^8$  CFU/mL。

水稻(武运粳 23)、小麦(宁麦 14)和玉米(伟科 966)种子表面灭菌处理(70%乙醇 1 min, 2%次氯酸钠溶液 5 min, 无菌水冲洗 5 次)后在湿润滤纸上 28 °C 黑暗条件下预发芽。2-3 d 萌动后, 在接种液(对照处理为无菌水)中浸泡 3 h<sup>[47-48]</sup>。挑选萌发状态一致的萌发种子种植在 Hoagland<sup>[48]</sup> 1%琼

脂平板上, 每个平板另加接种液(对照处理为无菌水) 1 mL, 在平板上加一层无菌水防止琼脂干裂, 28 °C 恒温光照培养箱中光暗交替(14:10)培养, 每个处理 3 次重复, 2 周后取样测定根长、株高、根干重和茎叶干重。

## 2 结果和分析

### 2.1 分离菌株的形态与生长

从固氮能力较强的紫色土发育水稻土富集培养物中分离得到菌株 P208。在固体无氮培养基上, 菌株 P208 菌落呈黏液状、表面光滑, 培养 5 d 后菌落中产生棕黑色非水溶性色素。菌株 P208 细胞形态呈卵圆形, 革兰氏阴性, 约(2–3)  $\mu\text{m} \times$  (4–5)  $\mu\text{m}$ , 常成对排列(图 1)。菌株 P208 在固体无氮培养基上生长较快, 在 28 °C 条件下划线培养 2 d 后即可长出稳定的单菌落, 1% 接种于液体无氮培养基中培养 18–20 h 后达到对数生长期(28 °C、180 r/min)。

### 2.2 16S rRNA 基因序列相似性及系统发育分析

菌株 P208 的 16S rRNA 基因序列(MK841645) 与 *A. chroococcum* IAM 12666<sup>T</sup>(=ATCC 9043<sup>T</sup>)

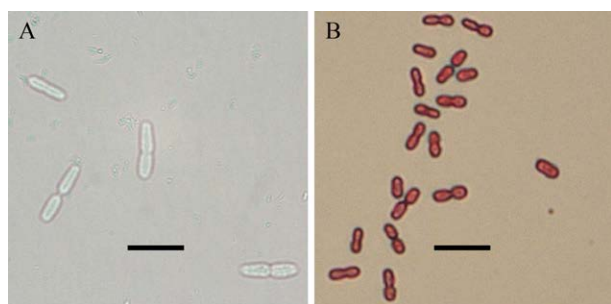


图 1. 菌株 P208 的细胞形态(Bar=10  $\mu\text{m}$ )

Figure 1. Cellular morphology of strain P208 (Bar=10  $\mu\text{m}$ ). A: phase-contrast microscopy; B: Gram staining.

(AB175653, 相似度为 99.79%)、*A. beijerinckii* ATCC 19360<sup>T</sup>(=DSM 378<sup>T</sup>) (AJ308319, 相似度为 98.66%) 具有相对较高相似度, 高于物种分类的阈值(>98.65%)<sup>[49]</sup>。与其他模式菌株 16S rRNA 基因序列相似度较低(<97.1%)。

基于 16S rRNA 基因的系统发育分析, 菌株 P208 与固氮菌属(*Azotobacter*) 7 个物种的模式菌株聚集形成 1 个独立的簇, 表明菌株 P208 应归于固氮菌属(*Azotobacter*) (图 2)。另外, 菌株 P208 与 *A. chroococcum* IAM 12666<sup>T</sup>(=ATCC 9043<sup>T</sup>) 系统发育距离最近, 其次为 *A. beijerinckii* ATCC 19360<sup>T</sup>(=DSM 378<sup>T</sup>)。

### 2.3 基因组核心基因系统发育分析

基于基因组 92 个核心基因的系统发育分析结果与 16S rRNA 基因具有一致性, 证实了菌株 P208 与 *A. chroococcum* ATCC 9043<sup>T</sup> 的系统发育距离最近, 且形成了 1 个区别于 *A. beijerinckii* DSM 378<sup>T</sup> 的独立分枝(GSI=90, 最大值为 92) (图 3)。

### 2.4 基因组特征与相关指数比较

菌株 P208 全基因组项目 GenBank/EMBL/DDBJ 登录号为 SWKB00000000 (表 1)。测序组装后得到的基因组大小为 5188530 bp, G+C 含量为 66.0 mol%。测序所得 Contig 247 个、N50 长度 49173 bp、测序深度 452 $\times$ , 采用 NCBI PGAP 注释共预测出 5067 个基因, 其中蛋白质编码基因 4714 个, RNA 基因 64 个, 伪基因 289 个。其中, 64 个 RNA 基因包括 rRNA 基因(5S/16S/23S) 6 个、tRNA 基因 54 个和 ncRNA 基因 4 个。通过对基因组注释结果中的固氮相关基因进行统计, 新分离菌株预测得到的固氮酶基因、固氮酶结构和功能相关基因有 32 个, 测序的基因组将为进一步研究功能基因、代谢途径和比较基因组学提供基础。

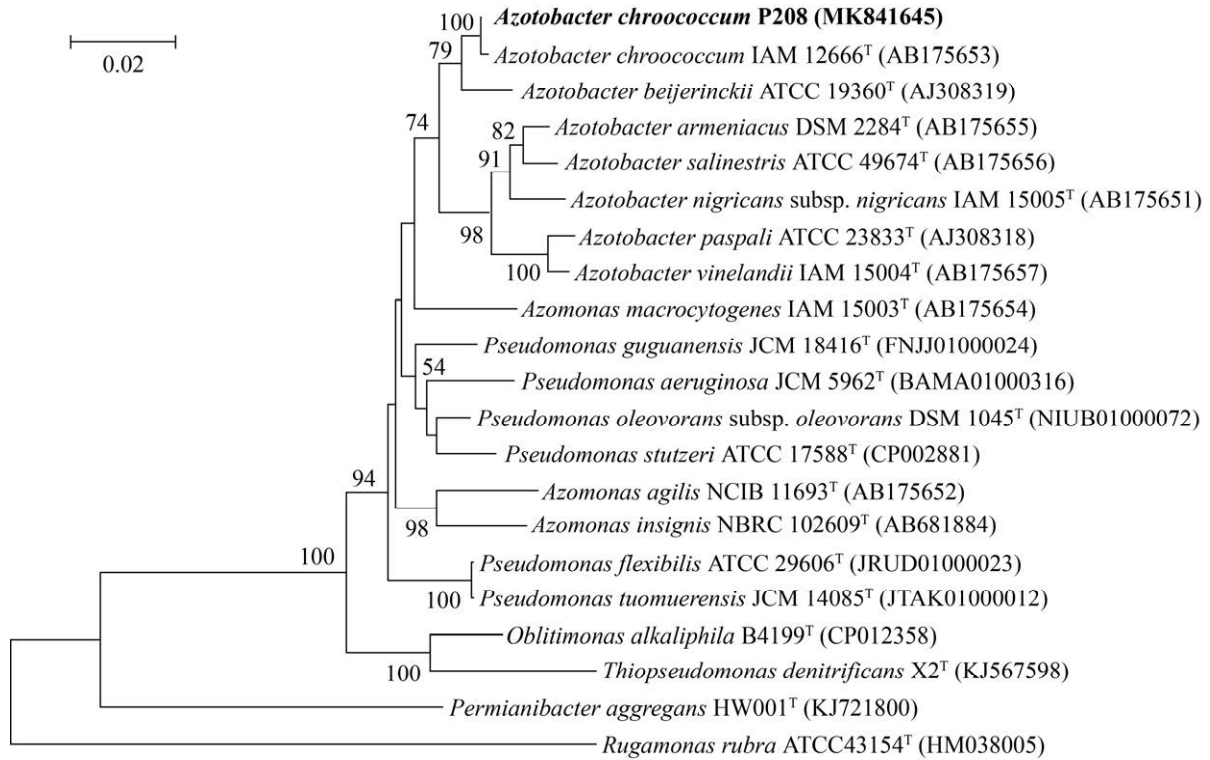


图 2. 基于 16S rRNA 基因的系统发育树

Figure 2. Phylogenetic tree based on 16S rRNA gene sequences.

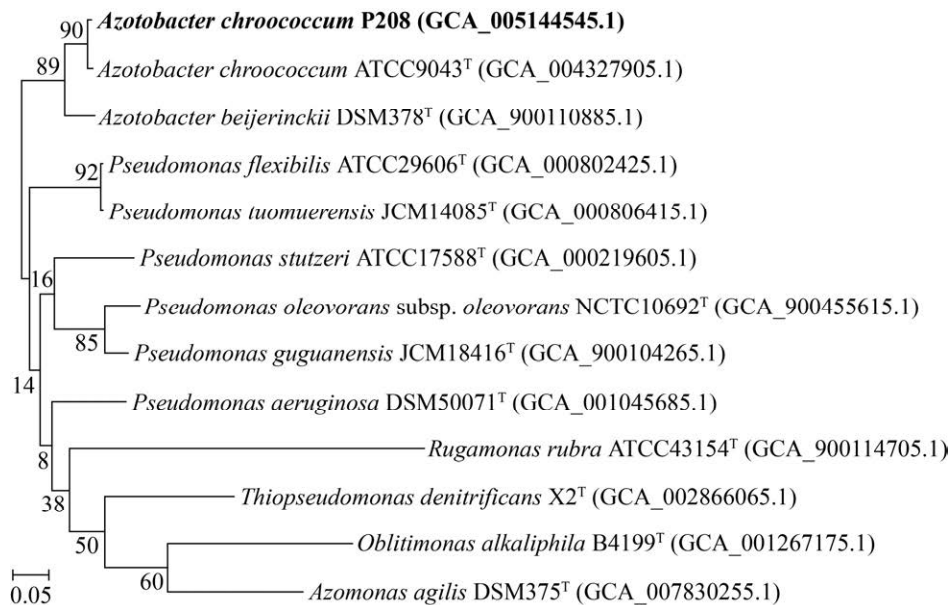


图 3. 基于基因组 92 个核心基因的系统发育树

Figure 3. Phylogenetic tree based on UBCGs (concatenated alignment of 92 core genes). Gene support indices (GSIs) values (maximal value is 92) are given next to the branches.

表 1. 菌株 P208 的基因组特征

Table 1. Genome properties of strain P208

Attributes	Values
GenBank accession	SWKB00000000
Genome size/bp	5188530
G+C content/(mol%)	66.0
Contigs	247
N50/bp	49173
Genome coverage/ $\times$	452
Total genes	5067
Protein-coding genes	4714
RNA genes	64
rRNA genes	6
tRNA genes	54
ncRNA genes	4
Pseudo genes	289

为进一步明确菌株 P208 在物种水平的系统分类地位,对其和密切相关的模式菌株 *A. chroococcum* ATCC 9043<sup>T</sup> (GCA\_004327905.1)、*A. beijerinckii* DSM 378<sup>T</sup> (GCA\_900110885.1)进行基因组相关指数比较(表 2)。结果表明,菌株 P208 与 *A. chroococcum* ATCC 9043<sup>T</sup> 的平均核苷酸一致性(ANI)、平均氨基酸一致性(AAI)和数字 DNA-DNA 杂交值(dDDH)均高于物种分类的阈值(ANI>95%–96%, AAI>95%–96%, dDDH>70%)<sup>[34,50–51]</sup>, 而与 *A. beijerinckii* DSM 378<sup>T</sup> 的 ANI、AAI、dDDH 均低于物种分类的阈值。菌株 P208 与 *A. chroococcum* ATCC 9043<sup>T</sup> 的最大唯一匹配指数(MUMi)低于物种分类的阈值(<0.33)<sup>[38]</sup>, 而与 *A. beijerinckii* DSM 378<sup>T</sup> 的 MUMi 值高于物种分类的阈值。同时,基因组 G+C 含量差异和四核苷酸频率相关系数(TETRA)均在同一物种的变异范围之内(分别为 <1%、>0.99)<sup>[50,52]</sup>。因此,结合其形态特征,菌株 P208 可鉴定为褐球固氮菌(*A. chroococcum*)。

表 2. 菌株 P208 与密切相关模式菌株的基因组比较

Table 2. Genome comparisons between strain P208 and closely related type strains

Genomic metrics	Strains	
	<i>Azotobacter chroococcum</i> ATCC 9043 <sup>T</sup>	<i>A. beijerinckii</i> DSM 378 <sup>T</sup>
ANI/%	96.82	89.87
AAI/%	96.05	89.19
dDDH/%	73.1–78.9	39.6–44.6
MUMi	0.268	0.583
Difference in G+C content/%	0.16	0.37
TETRA	0.9995	0.9971

Species circumscription thresholds: ANI (>95%–96%), AAI (>95%–96%), dDDH (>70%), MUMi (<0.33), difference in G+C content (<1%), TETRA (>0.99).

## 2.5 菌株的固氮能力

对数生长中期菌液在 10% 乙炔中培养 2 h 后,即可检测到较高的乙烯峰。定量结果显示, *A. chroococcum* P208 的乙烯生成速率显著高于模式菌株 *A. chroococcum* ATCC 9043<sup>T</sup> ( $P<0.01$ ) (图 4-A)。<sup>15</sup>N<sub>2</sub> 示踪法测定固氮活性的结果与乙炔还原法测定结果一致,菌株 P208 的固氮活性为 136.38 nmol N<sub>2</sub>/(mg protein·min), 约为模式菌株 *A. chroococcum* ATCC 9043<sup>T</sup> 的 2.61 倍(图 4-B)。

## 2.6 体外促生潜力

通过定量分析, *A. chroococcum* P208 具有 IAA 生成、溶磷活性和铁载体生成等促进植物生长潜力的培养特性(表 3)。*A. chroococcum* P208 的 IAA 和铁载体生成能力显著高于模式菌株 *A. chroococcum* ATCC 9043<sup>T</sup> ( $P<0.05$ )。

## 2.7 菌液接种对作物幼苗生长的影响

如表 4 所示,与未接种对照处理相比,接种 *A. chroococcum* P208 显著促进了水稻、小麦幼苗根系的生长( $P<0.05$ ),小麦根长增加 41.3%、根干

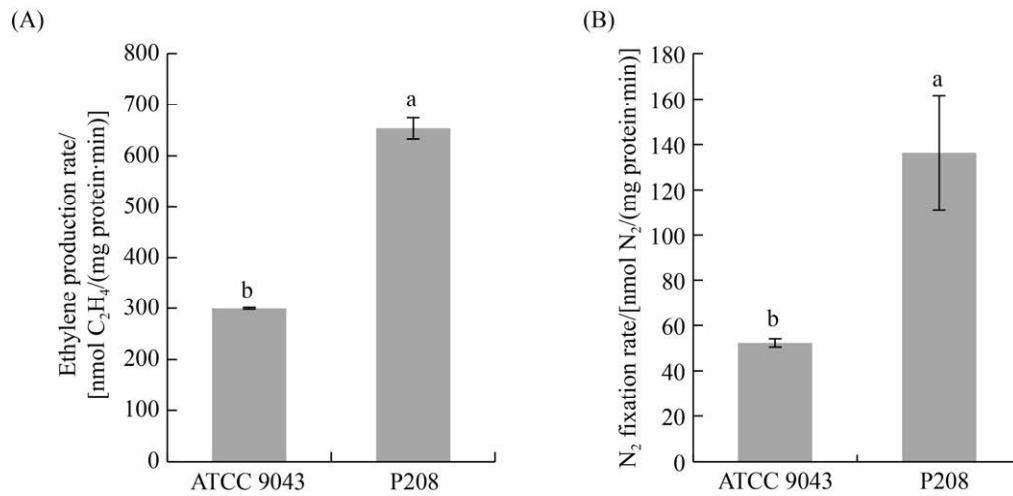


图 4. 新分离菌株与模式菌株的固氮能力

Figure 4. Nitrogen fixation activity of newly isolated strain and type strain. A: acetylene reduction assay; B: <sup>15</sup>N<sub>2</sub> tracer method. Error bars represent standard deviations ( $n=3$ ). The different letters above bars indicate significant differences at  $P<0.01$  level according to LSD.

表 3. 新分离菌株与模式菌株的体外促生潜力

Table 3. *In vitro* plant growth promotion potential of newly isolated strain and type strain

Strains	IAA/( $\mu\text{g}/\text{mL}$ )	Siderophore/%	Phosphate solubilization/( $\mu\text{g}/\text{mL}$ )
<i>Azotobacter chroococcum</i> ATCC 9043 <sup>T</sup>	12.47 $\pm$ 0.28 b	48.91 $\pm$ 7.26 b	1.29 $\pm$ 0.14 a
<i>A. chroococcum</i> P208	18.48 $\pm$ 0.48 a	88.21 $\pm$ 4.61 a	1.32 $\pm$ 0.16 a

Values are means $\pm$ standard deviations ( $n=3$ ). Data followed by the same letters in the same column were not significantly different at  $P<0.05$  level according to LSD.

表 4. *Azotobacter chroococcum* P208 接种对作物幼苗生长的影响

Table 4. Inoculation effects of *Azotobacter chroococcum* P208 on crop seedling growth

Crops	Treatments	Root length/cm	Shoot length/cm	Root dry weight/(mg/plant)	Shoot dry weight/(mg/plant)
Rice	Control	2.9 $\pm$ 0.6 b	12.4 $\pm$ 0.5 a	4.6 $\pm$ 0.4 b	11.9 $\pm$ 0.2 a
	Inoculation with P208	6.8 $\pm$ 0.9 a	12.2 $\pm$ 1.2 a	6.6 $\pm$ 0.5 a	11.0 $\pm$ 1.1 a
Wheat	Control	10.9 $\pm$ 1.6 b	17.7 $\pm$ 2.0 a	9.7 $\pm$ 0.8 b	29.3 $\pm$ 5.4 a
	Inoculation with P208	15.4 $\pm$ 1.0 a	19.2 $\pm$ 0.7 a	13.8 $\pm$ 0.9 a	31.8 $\pm$ 0.7 a
Maize	Control	18.7 $\pm$ 1.7 a	22.3 $\pm$ 1.9 a	67.1 $\pm$ 8.2 a	72.9 $\pm$ 3.0 a
	Inoculation with P208	18.2 $\pm$ 2.0 a	22.0 $\pm$ 1.1 a	67.3 $\pm$ 9.3 a	71.9 $\pm$ 11.5 a

Values are means $\pm$ standard deviations ( $n=3$ ). Data followed by the same letters in the same column for each crop were not significantly different at  $P<0.05$  level according to LSD.



重增加 42.3%，水稻幼苗根系生长受接种的影响更加显著，水稻根长增加 134.5%、根干重增加 43.5%。接种 *A. chroococcum* P208 对小麦、水稻幼苗的影响未体现在株高和茎叶干重的变化上。另外，玉米幼苗的生长指标在未接种对照和接种 *A. chroococcum* P208 处理间差异未达显著水平。

### 3 讨论

选择特定的生境和样品作为分离来源，有利于分离出特定功能的微生物菌株。在本研究中，从固氮能力较高的土壤样品中分离获得了固氮能力较高的 *A. chroococcum* P208，不同的培养基和培养条件能分离出不同的微生物菌株<sup>[53-54]</sup>，耐酸、耐盐、耐氨、耐氧压和碳源需求广的固氮微生物菌株能够增加菌株的接种成活率和预期效果，更多培养基和培养条件下的分离筛选工作还有待进一步进行。

16S rRNA 基因是编码原核生物一种 rRNA 的基因序列，基于其高度保守性和特异性，常被应用于原核生物的物种鉴定中<sup>[55]</sup>。随着测序技术的不断进步，基因组测序成本逐渐降低，在微生物分类鉴定中普遍采用基因组测序数据成为可能<sup>[56]</sup>。以平均核苷酸一致性(ANI)和数字 DNA-DNA 杂交值(dDDH)为代表的基因组相关指数(OGRI)逐渐成为原核生物物种划分的金标准<sup>[50]</sup>。实际操作中，甚至没必要测定鉴定菌株的完整基因组序列，随机测序 20%以上基因组进行分析就可以完成鉴定<sup>[50]</sup>。尽管 16S rRNA 基因保守性强，无法区分亲缘关系极近的菌株，但仍然是原核生物快速鉴定的重要方法，目前主要结合

16S rRNA 基因相似性比对和全基因组序列比较进行原核生物的物种鉴定，16S rRNA 基因序列相似性 97% (98.65%)<sup>[49,57]</sup>被广泛用作物种分类的阈值。固氮菌属(*Azotobacter*)物种之间 16S rRNA 基因的相似度分析结果(图 5)显示，固氮菌属物种的 16S rRNA 基因具有较高的保守性，*A. paspali* ATCC 23833<sup>T</sup>和 *A. vinelandii* IAM 15004<sup>T</sup>之间的 16S rRNA 基因相似度高达 98.97%。因此，16S rRNA 基因相似度 97% (甚至 98.65%)作为物种鉴定的阈值不适用于固氮菌属。在本研究中，菌株 P208 和 *A. beijerinckii* ATCC 19360<sup>T</sup>之间的 16S rRNA 基因相似度高于物种分类的阈值，但它们最终被归类为不同的物种。前人研究中，16S rRNA 基因序列相似度>99%的菌株也有分属于不同物种的可能<sup>[58-59]</sup>。该结果再次证明了 16S rRNA 基因相似度>97% (甚至 98.65%)进行物种水平鉴定的不确定性，以及全基因组序列比较(如 ANI、dDDH)对物种鉴定的必要性。

乙炔还原法是简单快速测定固氮能力的方法<sup>[60]</sup>，在使用中常通过系数  $R$  值( $R$ =乙烯生成速率/固氮速率)将乙炔还原活性换算为固氮活性，前人研究中多使用  $R=3$  作为理论换算系数<sup>[61]</sup>。本研究同时采用乙炔还原法和 <sup>15</sup>N<sub>2</sub> 示踪法测定菌株的固氮能力(图 4)，两种方法测定结果的换算系数  $R$  值为 4.90-5.74，高于理论  $R$  值。前人研究中，不同条件下测定的  $R$  值与理论  $R$  值也存在明显偏差，变化幅度从小于 1 到大于 30，大多数报道中  $R$  值为 2-7<sup>[40,62]</sup>。在大多数情况下，两种方法测定结果具备较好的一致性，而固氮速率较低时，测定结果差异较大<sup>[62]</sup>。因此，通过乙炔还原法定量测定固氮能力时，应同时结合 <sup>15</sup>N<sub>2</sub> 示踪法确定换算系数  $R$  值。

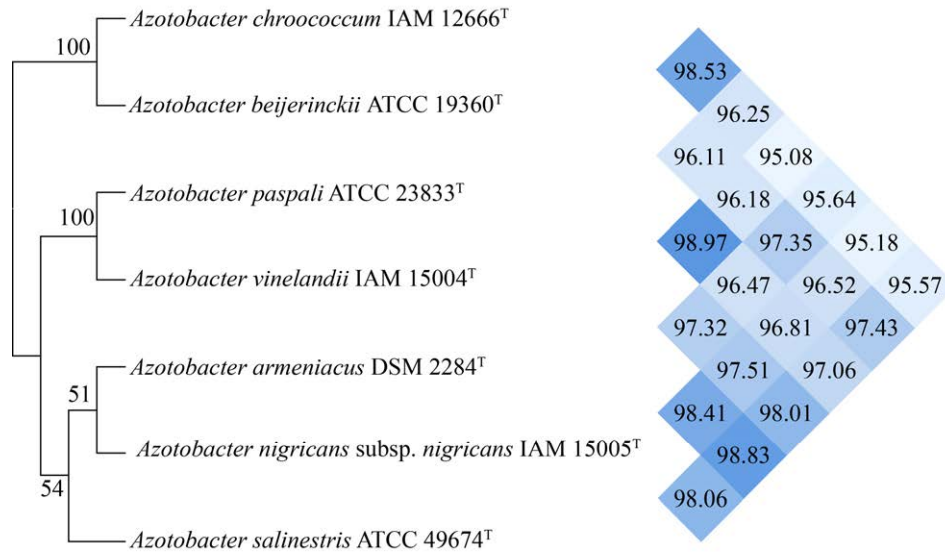


图 5. 固氮菌属物种之间 16S rRNA 基因的相似度与系统发育分析

Figure 5. Pairwise sequence similarities and phylogenetic analyses based on 16S rRNA gene of *Azotobacter* spp..

固氮促生菌在农业中的应用有助于减少农用化学品的使用,降低生产成本,增加经济效益和环境效益,利于生态农业的发展<sup>[63-65]</sup>。植物促生菌促进植物生长的机制主要包括:生物固氮、磷等元素的增溶和矿化、生长素等植物激素的生成、ACC 脱氨酶的生成、铁载体的生成和抗生素的生成等<sup>[66]</sup>。同一物种的不同菌株其促生潜力等特征存在显著差异<sup>[66]</sup>,而不同菌株的比较应在同一培养条件下进行,本研究选用模式菌株 *A. chroococcum* ATCC 9043<sup>T</sup> 作为参比,在同一条件下测定了新分离菌株 P208 的固氮能力及促生潜力(图 4,表 3),固氮活性为模式菌株的 2.61 倍,IAA 生成能力为模式菌株的 1.48 倍,铁载体生成能力为模式菌株的 1.80 倍,具有潜在的开发应用价值。前人研究表明,与溶磷作用等促生特征相比,IAA 和铁载体生成是植物促生菌筛选的优选特征,IAA 和铁载体生成有利于增强促生菌的环境适应性,促进菌株在植物根系的定殖<sup>[67]</sup>。菌株

生成的铁载体能够促进植物对铁的吸收,对植物病原微生物具有拮抗作用<sup>[68-69]</sup>;菌株 IAA 的生成与植株根茎生物量、氮磷吸收量显著正相关<sup>[70]</sup>,不同水平的 IAA 生成对植株根系伸长具有不同的效应,较低浓度的 IAA 生成与植株根系的伸长具有显著的正相关关系,而较高浓度的 IAA 生成抑制主根的伸长,促进侧根的生长,增加根系的生物量<sup>[67,70-71]</sup>。

为了解分离菌株的促生应用潜力,在室内培养条件下进一步研究了 *A. chroococcum* P208 接种对作物幼苗生长的影响,结果显示可显著促进水稻和小麦幼苗的根系生长(表 4)。更长、更丰富的根系可能促进植物对水分和养分的吸收<sup>[48]</sup>。然而,结果中未检测到接种对茎叶生长的显著影响,这可能归因于较短的培养时间和 Hoagland 植物生长培养基中丰富的营养。在相同的试验条件下,玉米幼苗根系和茎叶的生长均对接种没有明显的响应,这表明 *A. chroococcum* P208 接种对

不同作物幼苗生长的影响不同。前人研究表明, 不同类型的农作物品种对细菌接种的反应不同<sup>[7,66]</sup>。

*A. chroococcum* P208 接种对玉米幼苗生长的影响较小也可能归因于供试材料的基因型。因此, 涉及更多作物和品种的接种试验研究有待进一步开展。

综上, 本研究从固氮能力较高的紫色土发育水稻土中分离到一株具有较强固氮促生能力的固氮菌, 利用基于基因组的方法鉴定为褐球固氮菌 (*Azotobacter chroococcum*), *A. chroococcum* P208 能够为进一步的生物技术提供新的固氮酶、固氮酶基因和代谢途径。此外, 作为一株高效固氮促生菌株资源, *A. chroococcum* P208 的应用前景值得进一步探索。

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# Genome-based identification and plant growth promotion of a nitrogen-fixing strain isolated from soil

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**Abstract:** [Objective] The aim of this study is to obtain efficient nitrogen-fixing strains and fully utilize soil nitrogen-fixing microbial resources. [Methods] The Entisol with superior nitrogen fixation capability was selected to isolate nitrogen-fixing strains via enrichment and purification procedure. The identification of newly isolated strain at species level was performed by means of 16S rRNA gene phylogenetic analysis and whole genome correlation index comparison. Quantitative determination of nitrogen fixation activity of newly isolated strain was performed by acetylene reduction assay and <sup>15</sup>N<sub>2</sub> tracer method. The plant growth promotion potentials of the nitrogen-fixing strain were preliminarily determined through culture characteristics and inoculation effects. [Results] A strain, designated P208, which could grow rapidly in nitrogen-free medium, was isolated from Entisol. Phylogenetic analyses based on 16S rRNA gene and 92 core genes revealed that strain P208 was most phylogenetically closely related to *Azotobacter chroococcum* IAM 12666<sup>T</sup> (=ATCC 9043<sup>T</sup>) (99.79% 16S rRNA gene similarities). The average nucleotide identity (ANI), average amino acid identity (AAI) and digital DNA-DNA hybridization (dDDH) values based on genome sequences of strain P208 and *A. chroococcum* ATCC 9043<sup>T</sup> were higher than the thresholds for species circumscription (ANI>95%–96%, AAI>95%–96%, dDDH>70%), and the maximal unique matches index (MUMi) value was lower than the species circumscription threshold (<0.33). Therefore, The newly isolated strain P208 could be identified as *A. chroococcum*. The nitrogen fixation activity of *A. chroococcum* P208 was 2.61 times that of the type strain *A. chroococcum* ATCC 9043<sup>T</sup>. In addition to nitrogen fixation, *A. chroococcum* P208 had plant growth promotion potentials in cultures, such as IAA production, phosphate solubilization and siderophore production. Inoculation with *A. chroococcum* P208 could promote root growth of rice and wheat seedlings under controlled conditions. [Conclusion] A strain with superior nitrogen fixation and plant growth promotion potential was isolated and purified from the paddy soil with superior nitrogen fixation capability. It has relatively good development and application potential value, and could provide microbial resource for the study and utilization of biological nitrogen fixation.

**Keywords:** biological nitrogen fixation, nitrogen-fixing bacteria, *Azotobacter chroococcum*, plant growth promotion, plant growth-promoting bacteria

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