益生蜡样芽孢杆菌可转移四环素耐药基因 $tet(45)$ 特征分析

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摘要 蜡样芽孢杆菌作为益生菌常用菌种之一被广泛应用于人类医疗保健、畜禽养殖、作物促生长和生物防控、环境水质净化等多个领域。益生菌携带的可转移性耐药基因对公共卫生构成潜在威胁。本团队前期发现1株分离自动物源微生态制剂的蜡样芽孢杆菌(*Bacillus cereus* 9i, BC 9i)携带可转移的四环素耐药基因。通过生物信息学分析发现, BC 9i携带的四环素耐药基因为 $tet(45)$ 基因变体, 同属于膜转运蛋白超家族基因。BC 9i表达的Tet(45)变体蛋白结构与原Tet(45)蛋白结构类似, 均由14个跨膜螺旋核心构成, 但在环状区域和 α -螺旋中表现出细微差异。通过克隆 $tet(45)$ 基因变体, 研究表明其功能是通过四环素外排泵介导耐药性产生, 外排泵抑制剂可以有效抑制该作用。本研究从基因、蛋白和功能等方面揭示可转移 $tet(45)$ 基因变体的特征, 为探究益生菌耐药基因来源以及益生菌候选菌株的筛选和合理使用提供科学依据。

关键词 益生菌, 蜡样芽孢杆菌, 细菌耐药性, 可转移耐药基因, 四环素

细菌耐药性是当前关注的主要公共卫生问题之一, 抗生素耐药基因被视为新型的环境污染物, 在全世界引起广泛关注^[1]。但抗生素耐药基因是古老的, 这已通过远古冻土样品中抗生素耐药基因的鉴定得到证实^[2]。如今, 抗生素的过度使用和滥用使细菌长期处于选择性压力之下, 因而增强了细菌耐药性。此外, 抗生素耐药基因的突变率增加, 并加之可转移耐药基因的积累, 都加剧了细菌耐药性的产生^[3,4]。例如, 编码外排泵蛋白是细菌对四环素产生耐药性的主要作用机制^[5]。当前已鉴定出35种编码四环素外排泵蛋白基因(<http://faculty.washington.edu/marilynr/>, 更新于2020年2月20日), 而在2012年这一值为28^[6]。

为缓解日益严峻的耐药性问题, 益生菌作为一种对宿主有益的抗生素有效替代品, 被广泛应用于人类医疗保健、畜禽养殖、作物促生长和生物防控、环境水质净化等各个方面^[7,8]。蜡样芽孢杆菌次级代谢产物丰富, 是益生菌常用菌种之一。1989年美国食品药品监督管理局(Food and Drug Administration, FDA)与饲料工业协会(America Feed Industry Association, AFIA)就允许蜡样芽孢杆菌作为饲料添加剂应用于畜牧生产中^[9]。目前, 益生菌的总体使用记录显示出较令人满意的安全性结果, 但相对于其应用的广泛度来说, 益生菌的安全性并未受到充分鉴定^[10,11]。本团队前期对益生芽孢杆菌的毒性和耐药性进行了系统评估, 发现菌株

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耐药问题较为严重^[12]. 其中分离到1株携带四环素耐药基因的蜡样芽孢杆菌分离株9i(*Bacillus cereus* 9i, BC 9i), 该基因与四环素外排泵基因 $tet(45)$ 显示出高相似度, 位于转座元件并具备转移性^[13]. 最初发现 $tet(45)$ 基因位于1株分离自家禽养殖场土壤样本的*Bhargavaea cecembensis*菌株染色体上, 编码的458个氨基酸与四环素外排泵蛋白Tet(L)共享78%的一致性^[14]. BC 9i分离自用于动物饲料添加的微生物制剂, 其携带的可转移四环素耐药基因 $tet(45)$ 与报道的 $tet(45)$ 基因在序列和遗传环境上均表现出明显差异. 随着我国“减抗/替抗”政策的实施, 益生菌在畜牧生产中使用愈加广泛. 因此, 十分有必要对BC 9i所携带的可转移四环素耐药基因 $tet(45)$ 特征进行全面分析.

本研究以分离自动物源微生物制剂的蜡样芽孢杆菌BC 9i为研究对象, 分析其携带的可转移四环素耐药基因 $tet(45)$ 的特征, 包括从基因和蛋白水平进行多重比较, 解析系统发生关系, 并初步探究其耐药作用机制, 以便更好地了解耐药基因在益生菌株中出现和传播扩散风险, 为后期筛选和安全有效使用益生菌提供科学依据.

1 材料与方法

1.1 $tet(45)$ 基因特征分析

BC 9i分离自动物饲料添加的微生物制剂. 通过Illumina HiSeq 2500系统测序获得BC 9i的全基因组序列, 使用CLC Genomics Workbench 9.5.3工作平台对全基因组序列进行拼装, 共获得26个平均长度为203093 bp的拼接序列, 平均覆盖度为411, 测序序列拼接效果良好, BC 9i的全基因组序列可用于后续分析. BC 9i的全基因组序列已提交至NCBI数据库, 生物样品编号为SAMN07267335. 其携带的可转移四环素耐药基因 $tet(45)$ 已上传至GenBank数据库, 基因序列编号为KX091844.1.

通过四环素耐药基因数据库(<http://faculty.washington.edu>)和NCBI核酸序列数据库(<https://www.ncbi.nlm.nih.gov/nucleotide/>)检索其他四环素外排泵类耐药基因信息, 汇总如表S1所示. 使用ClustalX 2.1.9对选定序列进行多重比对, 使用MEGA 7.0.20软件, 采用Kimura 2-parameter参数模型和Neighbor-joining算法构建系统发生树, 并通过1000次重复bootstrap方法评估所得树的可靠性^[15,16]. 最后, 通过iTOL在线软件可视化系统发生树^[17].

SWISS-MODEL软件用于蛋白质3D结构预测^[18]. 使用*Geobacillus kaustophilus*菌株表达的同属于膜转运蛋白超家族的质子依赖性肽转运蛋白的晶体结构(PDB ID 4ikv)作为结构预测模板^[19]. 最后使用UCSF Chimera 1.11.2.14软件生成蛋白质3D结构图, 并进行结构差异分析^[20].

1.2 $tet(45)$ 基因功能分析

通过ClonExpress克隆试剂盒将来自BC 9i的四环素耐药基因 $tet(45)$ 克隆到穿梭质粒pAM401中, 按照美国临床实验室标准化协会建议, 通过经典的微量肉汤稀释法检测原始菌株和携带该重组穿梭质粒的*Staphylococcus aureus* RN4220转化菌株对四环素的最小抑菌浓度, 以验证 $tet(45)$ 基因功能^[21]. *S. aureus* ATCC 29213作为质量控制菌株. 此外, 添加不同种类的外排泵抑制剂羰基氰化物间氯苯腈(carbonyl cyanide *m*-chlorophenylhydrazone, CCCP)、苯丙氨酸-精氨酸 β -萘胺(Phe-Arg- β -naphthylamide, P β N)和利血平(reserpine)检测 $tet(45)$ 基因功能.

2 结果与讨论

2.1 $tet(45)$ 基因系统发生分析

前期研究发现, $tet(45)$ 基因与四环素外排泵编码基因 $tet(L)$ 序列相似度为78%^[14]. 本研究进一步对BC 9i的全基因组序列进行分析, 确证携带的 $tet(45)$ 基因位于1个可移动的遗传元件上, 其基因环境如图1(a)所示. BC 9i携带的 $tet(45)$ 基因与报道的 $tet(45)$ 基因(GU584217.2)序列相似度为90%, 与 $tet(L)$ 基因(NC_010875.1)序列的相似度为81%. 参照新耐药基因命名建议^[22], 基因序列差异大于2%但小于20%, BC 9i携带的四环素耐药基因为 $tet(45)$ 基因变体.

我们从不同数据库收集了四环素外排泵型耐药基因序列, 以分析BC 9i中 $tet(45)$ 基因变体的系统发生关系. 结果如图1(b)所示, $tet(45)$ 基因变体在进化关系上与 $tet(45)$ 基因相似, 都与 $tet(L)$ 基因最为相近, 因此在功能上应同属于第2组的四环素外排泵型耐药基因. 但对BC 9i中 $tet(45)$ 基因变体所在的基因环境分析发现, $tet(45)$ 基因变体位于可移动元件上, 被不同的整合酶基因所包围(图1(a)), 这与原 $tet(45)$ 基因环境有较大差异(最初发现位于*Bhargavaea cecembensis*菌株染色体上). 综合 $tet(45)$ 基因变体与 $tet(45)$ 原基因在系统发生关系上的

图 1 BC 9i中*tet(45)*基因特征。(a) 细菌对四环素耐药机制主要包括通过外排泵主动外排、通过核糖体保护作用、对四环素酶解作用, 以及改变作用靶点。BC 9i中携带的四环素耐药基因*tet(45)*位于可移动元件, 基因环境如图所示。(b) BC 9i携带的*tet(45)*基因与其他四环素外排泵型耐药基因的系统发生关系

Figure 1 Characteristics of *tet(45)* in BC 9i. (a) The mechanism of bacterial resistance to tetracycline mainly includes efflux pumps, ribosomal protection, enzymatic inactivation and target modification. The *tet(45)* gene in BC 9i is located in a mobile element, and its genetic environment is shown in the figure. (b) Phylogeny of *tet(45)* in BC 9i and other tetracycline efflux pump genes

相似性与基因环境的差异, 我们推测*tet(45)*基因变体来源于四环素选择性压力诱导, 但其基因的可移动性来源仍需进一步研究。

益生菌携带可移动的耐药基因, 会随着益生菌的广泛使用而传播扩散, 并在环境中蓄积, 这对公共健康构成了极大的潜在风险。2002年联合国粮食及农业组织(Food and Agriculture Organization of the United

Nations, FAO)和世界卫生组织(World Health Organization, WHO)发布的益生菌评估指南中明确规定, 确定无可转移的耐药性是筛选益生菌候选株的先决条件之一^[23]。例如, 作为动物饲料添加使用的蜡样芽孢杆菌EsporaFeedPlus[®]因携带可移动的四环素耐药基因*tet(B)*, 欧洲食品安全局撤销了其销售许可^[24]。因此, 对于携带可移动耐药基因元件菌株的使用, 如BC 9i需要重新评

估, 筛选益生菌候选菌株时应避免携带可以转移耐药基因元件等潜在风险因子, 获得遗传稳定性高的候选菌株.

2.2 Tet(45)蛋白3D结构预测

我们预测了 *tet(45)* 基因及 BC 9i 中 *tet(45)* 基因变体的蛋白质3D结构, 从蛋白水平比较两者之间差异. 结果如图2(a)所示, BC 9i 中的 Tet(45) 氨基酸序列与原 Tet(45) 序列的相似性为88%. 两蛋白均由14个跨膜 α -螺旋组成的通道结构为主体, 这与膜转运蛋白超家族蛋白结构类似^[25], 但两者结构仍存在部分差异, 如图2(b)所示. Tet(45) 与 Tet(45) 变体环状区域存在差异, 包括第174~188、201~211、231~250和402~431位氨基酸残基

差异. 同时, Tet(45) 变体结构包含更多疏水性的 α -螺旋, 如Pro233-Phe234-Ile235和Pro238-Ser239-Leu240, 但这些差异结构对蛋白功能的影响仍有待检测.

2.3 *tet(45)* 基因外排功能

我们从 BC 9i 中克隆了 *tet(45)* 基因变体, 以进一步检测其功能. 通过对四环素类药物的最小抑菌浓度检测, BC 9i 及其克隆转化株 *S. aureus* RN4220/pAM401-*tet(45)* 显示出对四环素类(包括四环素、多西环素、土霉素和金霉素)药物的耐药性(表1).

此外, 为验证 *tet(45)* 基因变体的外排功能, 我们检测了 *tet(45)* 基因变体克隆转化菌株 *S. aureus* RN4220/pAM401-*tet(45)* 在不同外排泵抑制剂作用下对四环素

图2 Tet(45)变体与原Tet(45)蛋白水平差异. (a) BC 9i 中的 Tet(45) 变体氨基酸序列与原 Tet(45) 氨基酸序列对比. (b) BC 9i 产生的 Tet(45) 变体蛋白(红)与原 Tet(45) 蛋白(灰)3D 结构差异

Figure 2 Differences between Tet(45) variants and original Tet(45) in protein level. (a) Comparison of the amino acid sequence of Tet(45) variant in BC 9i with the original Tet(45). (b) Comparison of the predicted protein 3D structures of Tet(45) variant in BC 9i against the original Tet(45). The differences are highlighted in red

表1 *tet(45)* 基因变体克隆相关菌株对四环素类药物最小抑菌浓度(mg/L)^{a)}

Table 1 Minimum inhibitory concentrations of tetracyclines of *tet(45)* gene variant cloning related isolates

菌株	四环素	多西环素	土霉素	金霉素
<i>B. cereus</i> 9i	16	16	32	32
<i>S. aureus</i> RN4220	1	1	4	2
<i>S. aureus</i> RN4220/pAM401- <i>tet(45)</i>	16	16	32	32

a) *B. cereus* 9i 为 *tet(45)* 基因变体克隆供体菌株, *S. aureus* RN4220 为受体菌株, *S. aureus* RN4220/pAM401-*tet(45)* 为携带 *tet(45)* 基因变体克隆的受体菌株

的敏感性,结果如图3所示.不同类型的外排泵抑制剂均可提高 $tet(45)$ 基因变体克隆转化株对四环素的敏感性,其中利血平的作用效果最为明显,表明 $tet(45)$ 基因变体能够编码膜转运蛋白超家族外排蛋白,进而耐受四环素类药物.

3 结论

总的来说,本研究通过对四环素耐药基因 $tet(45)$ 变体的系统发生分析、蛋白结构预测及功能检测,推测BC 9i所携带的 $tet(45)$ 基因变体可能来源于环境选择性压力诱导 $tet(L)$ 基因突变.通过编码膜转运蛋白超家族的跨膜蛋白,主动将药物泵出胞外,进而获得对四环素类药物的耐受性.但 $tet(45)$ 基因变体与 $tet(L)$ 基因是否存在功能差异,以及 $tet(45)$ 基因变体是如何整合到转座元件获得移动性等问题仍需进一步研究.同时,对携带可转移耐药基因的益生菌使用需重新考量,对益生菌的筛选与安全性评估应当引起重视.

图3 外排泵抑制剂对 $tet(45)$ 基因变体功能的影响.测试菌株为*S. aureus* RN4220电转化菌株,其携带具有 $tet(45)$ 基因克隆的质粒pAM401(即*S. aureus* RN4220/pAM401- $tet(45)$).TET,四环素;CCCP,羰基氰化物间氯苯胺;PAβN,苯丙氨酸-精氨酸β-萘胺;Reserpine,利血平

Figure 3 Effects of efflux pump inhibitors on the function of $tet(45)$ gene variant. The tested strain is *S. aureus* RN4220 transformant carrying the plasmid pAM401 with the cloned $tet(45)$ gene variant (the strain *S. aureus* RN4220/pAM401- $tet(45)$). TET, tetracycline; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PAβN, Phe-Arg-β-naphthylamide

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Summary for “益生蜡样芽孢杆菌可转移四环素耐药基因 $tet(45)$ 特征分析”

Characteristics of a transferable $tet(45)$ gene conferring resistance to tetracyclines in probiotic *Bacillus cereus*

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Probiotic microbes conferring health benefits to the hosts have attracted great concerns recently. Particularly, spore-forming *Bacillus cereus* (*B. cereus*), Gram-positive, aerobic or facultative anaerobic bacteria, have been widely used in probiotic preparations for different purposes, due to their high stability to harsh conditions. Probiotics are generally recognized as safe (GRAS) and showing satisfactory overall safety records till now. However, the safety of each probiotic isolate is not guaranteed. The verification of the absence of mobile antimicrobial resistance genes and virulence factors is a prerequisite for the approval of probiotic candidates, according to the guidelines suggested by the Food and Agriculture Organization (FAO) and World Health Organization (WHO). The role of *B. cereus* and other members of the *B. cereus* group in gastrointestinal and non-gastrointestinal diseases has not always gained sufficient attention in the past, particularly in view of their use as probiotics. Therefore, the presence of transferrable antimicrobial resistance genes will endow probiotics as a reservoir with increased ability of emergence and dissemination, during their wide and inadvertent use.

Our previous study found that the presence of transferable tetracycline resistance gene $tet(45)$ in a probiotic isolate *B. cereus* 9i (BC 9i) derived of animal feed additive. The aim of the present work was to systematically characterize this $tet(45)$. The draft assembled genome of BC 9i was first used to analyze the genetic environment of $tet(45)$. Meanwhile, multiple alignment and a maximum likelihood tree were built to conduct the phylogenetic relationship between $tet(45)$ and other tetracycline efflux resistance genes. In addition, the 3D protein structures were predicted to identify differences between Tet(45) and the original Tet(45). Lastly, the $tet(45)$ gene in BC 9i was cloned and transferred into *Staphylococcus aureus* RN4220 to the minimum inhibitory concentrations, in the presence and absence of classic inhibitors of efflux pumps.

The phylogenetic analysis showed that the gene $tet(45)$ in BC 9i was classified as a gene variant of $tet(45)$, belonging to the major facilitator superfamily of membrane transporters. Additionally, the predicted structure of Tet(45) variant was similar to the original Tet(45), both sharing a 14-transmembrane helix core, although they exhibited subtle structural differences in the loop regions and α -helices. Lastly, the resistance to tetracyclines in $tet(45)$ variant was suppressed in the presence of efflux pump inhibitors. These results indicated that the $tet(45)$ variant in BC 9i encoded a efflux pump against tetracyclines.

Collectively, our findings suggest that such probiotic isolates harboring transferable antimicrobial resistance genes may consist a potential risk for public health. It sheds light on the subsequent screening of probiotic candidates and the appropriate application of probiotics for various purposes, to promote and improve One Health.

probiotic, *Bacillus cereus*, antimicrobial resistance, transferable resistance gene, tetracycline

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