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专论与综述

乳酸菌的基因组编辑技术研究进展

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摘　要：乳球菌(*Lactococcus* sp.)和乳杆菌(*Lactobacillus* sp.)是工业上常用的乳酸菌(lactic acid bacteria, LAB)，长期应用于食品和饮料的发酵。近年来，随着分子操作及遗传改造技术的不断完善，推动了乳球菌和乳杆菌的基础和应用研究，其作为功能菌株和工业微生物细胞工厂的重要潜能也不断突显出来。本文综述了工业常用乳酸菌的基因组编辑技术研究进展，着重介绍了基于整合质粒的敲除、敲入，基于基因组重组工程的精细修饰和敲除、敲入，以及基于成簇的规律性间隔的短回文重复序列及其相关蛋白 9 [(clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease9 (Cas9), CRISPR/Cas9]系统的基因组编辑技术。
关键词：乳球菌；乳杆菌；整合质粒；基因组重组工程；CRIPSR/Cas9 系统

Genome editing technology of lactic acid bacteria

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Abstract: *Lactococcus* sp. and *Lactobacillus* sp. are lactic acid bacteria commonly used in industry and have long been used in food and beverage fermentation. In recent years, with the

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continuous improvement of molecular manipulation and genetic modification techniques, the basic research and application of *Lactococcus* and *Lactobacillus* have been promoted, and their important potential as functional strains as well as industrial microbial cell factories has been highlighted. In this paper, the research progress of genome editing technology of lactic acid bacteria commonly used in industry was reviewed, and the knockout knock-in based on integrative plasmid, fine modification and knockout knock-in based on genome recombination engineering, and gene editing of CRIPSR/Cas9 system based on clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated nuclease9 (Cas9) were emphatically introduced.
Keywords: *Lactococcus* sp.; *Lactobacillus* sp.; integrative plasmids; genome recombination engineering; CRIPSR/Cas9 system

乳酸菌(lactic acid bacteria, LAB)是一组无芽孢、兼性厌氧、碳水化合物代谢产物主要以乳酸为主的一类细菌。LAB 种类繁多，在遗传组成和生理代谢方面具有高度多样性[1]，其中乳球菌属和乳杆菌属在遗传代谢和基因工程领域研究较多[2-5]。作为公认安全级(generally recognized as safe, GRAS)菌株，发酵食品是乳酸菌最重要的经济应用之一，商业化使用时间最长，预计未来 5 年市场增长 7.2%[6]。近年来，随着基因工程和合成生物学技术的发展，提升乳酸菌潜在的生产性能、安全性及稳定性并构建乳酸菌细胞工厂受到了工业界和医学界的广泛关注[7-8]。

由于乳酸菌的重要应用价值及潜力，越来越多的研究致力于构建适用于该菌株的遗传改造工具和方法。20 世纪 80 年代，基于异源转座子和原噬菌体整合酶等方法在乳酸菌中实现了染色体整合和基因失活[9-11]。21 世纪初，第 1 个乳酸菌基因组序列(*Lc*. *lactis* subsp. *lactis* IL1403)被公布[12]，为实现乳酸菌遗传背景解析和定向基因组改造奠定了基础。随着基因组学和分子生物学的快速发展，研究者对乳酸菌的遗传操作系统如质粒复制系统、异源表达的启动子系统、选择系统、表达系统、转化技术等不断完善，促进了乳酸菌基因组工程技术的开发和优化，进一步推动了乳酸菌应用和基础研究[13-14]。目前，乳酸菌作为工业重要的微生物细胞工厂已成功用于高附加值化合物、抗菌剂、甜味剂和重要工业酶的生产[6,15]。除此之外，乳酸菌还被开发作为药物和疫苗的载体用于生物治疗、预防和诊断方面的医疗应用[16-18]。本文总结了针对工业上常用的乳球菌和乳杆菌开发的基于整合质粒、基因组重组工程及基于成簇的规律性间隔的短回文重复序列及其相关蛋白 9 [(clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nuclease9 (Cas9), CRISPR/Cas9]系统的基因组编辑技术(表 1)，并概述了其在构建乳酸菌细胞工厂中的应用。

1 基于整合质粒的敲除、敲入

目前，常用的乳酸菌基因组编辑技术主要是依赖细胞内源重组蛋白 RecA 介导的整合型质粒与基因组发生的同源双交换事件。由于 RecA 依赖性的双交换频率低，阳性克隆子筛选周期长，研究者开发了一系列非复制型[19,34]和条件复制型[21,35]整合质粒结合反向筛选标记[36-38]，进一步提高了该系统的整合和筛选效率。

1.1 整合质粒

整合质粒在很多微生物的基因组编辑技术发展历程中都发挥着不可或缺的作用，载有筛选标记基因和基因组靶标基因上、下游同源臂

表 1 乳球菌和乳杆菌中可用的基因组编辑技术

Table 1 Genome editing techniques available in *Lactococcus* and *Lactobacillus*

Tools	Host range	Characteristics	References
Integrative plasmid	*Lc. Lactis*, *Lb. plantarum*, *Lb. rhamnosus*, *Lb. acidophilus*, *Lb. gasseri*, *Lb. casei*	Based on two rare single crossing-over, low integration efficiency and time consuming; No resistance markers; dependent on counter-screening markers	[19-21]
ssDNA recombination	*Lb. reuteri*, *Lc. lactis*, *Lb. plantarum*, *Lb. gasseri*	Recombinase mediated, suitable for genome fine modification, low recombination efficiency; Requires high concentration of oligonucleotides and mismatch repair (MMR) system; No resistance markers	[2-3]
dsDNA recombination	*Lb. plantarum*, *Lb. casei*, *Lb. paracasei*	Recombinase mediated, suitable for gene deletion and insertion; Combined with Cre/*loxP* system to cause *lox*72 residue; No resistance marker	[22-23]
CRIPSR/Cas9	*Lb. reuteri*, *Lc. lactis*, *Lb. plantarum*, *Lb. brevis*	Combined with ssDNA or dsDNA recombination, recombination before cleavage, providing efficient counter-screening tools; Recombinase mediated, cleavage before recombination, enabling gene deletion insertion and fine modification; No resistance markers	[24-28]
CRISPR/Cas9^{D10A}	*Lb. casei*	Suitable for deletion and insertion of genes; No resistance marker; Cas9^{D10A} causes genomic single-strand DNA breaks, which is easier to repair and can effectively avoid high mortality caused by double-strand breaks (DSBs) and is suitable for strains with low recombination capacity	[29]
CRIPSR/dCas9	*Lc. lactis*, *Lb. plantarum*	Specific targeting of single or multiple target genes using dCas9 without endonuclease activity for downregulation at the mRNA level; No resistance marker	[30-32]
CRISPR/DBE	*Lc. lactis*	CRISPR-deaminase-assisted base editor, including CRISPR/cDBE and CRISPR/aDBE; Specifically targeted by sgRNA, can efficiently achieve base editing; No resistance marker	[33]

的整合质粒，在细胞自身 RecA 作用下实现与基因组同源区域的两轮重组交换[20,39]。如图 1 所示，在第一轮交换中，质粒上的上游同源臂与基因组同源区进行交换，将整个质粒整合到基因组上，实现载体基因的复制，同时赋予重组菌株抗性标记。第二轮交换中，下游同源臂与基因组同源区进行交换，导致靶标基因、筛选标记基因及整合质粒上其他元件从基因组上去除。最终整合质粒上携带的上、下游同源臂取代靶标基因及其上、下游同源序列，实现基因的无痕敲除。目前乳酸菌中常用的非复制型整合质粒主要由 pACYC184[40]、pUC[34]和 pBluescript SK^{-}[41]等衍生而来。例如，宿主范围比较广的突变载体 pNZ5319 (衍生于pACYC184)被证明适用于乳酸菌的自杀突变，目前已广泛用于乳酸乳球菌(*Lactococcus lactis*, *Lc. Lactis*)和植物乳杆菌(*Lactobacillus plantarum*, *Lb. plantarum*)中基因的敲除和敲入[19-20]。张雅婷[42]通过对基于突变载体pNZ5319的基因组编辑方法进行优化，以 *Lc. lactis* NZ9000-3 为出发菌株构建了一系列基因组敲除菌株，为乳球菌合成生物学底盘的构建奠定

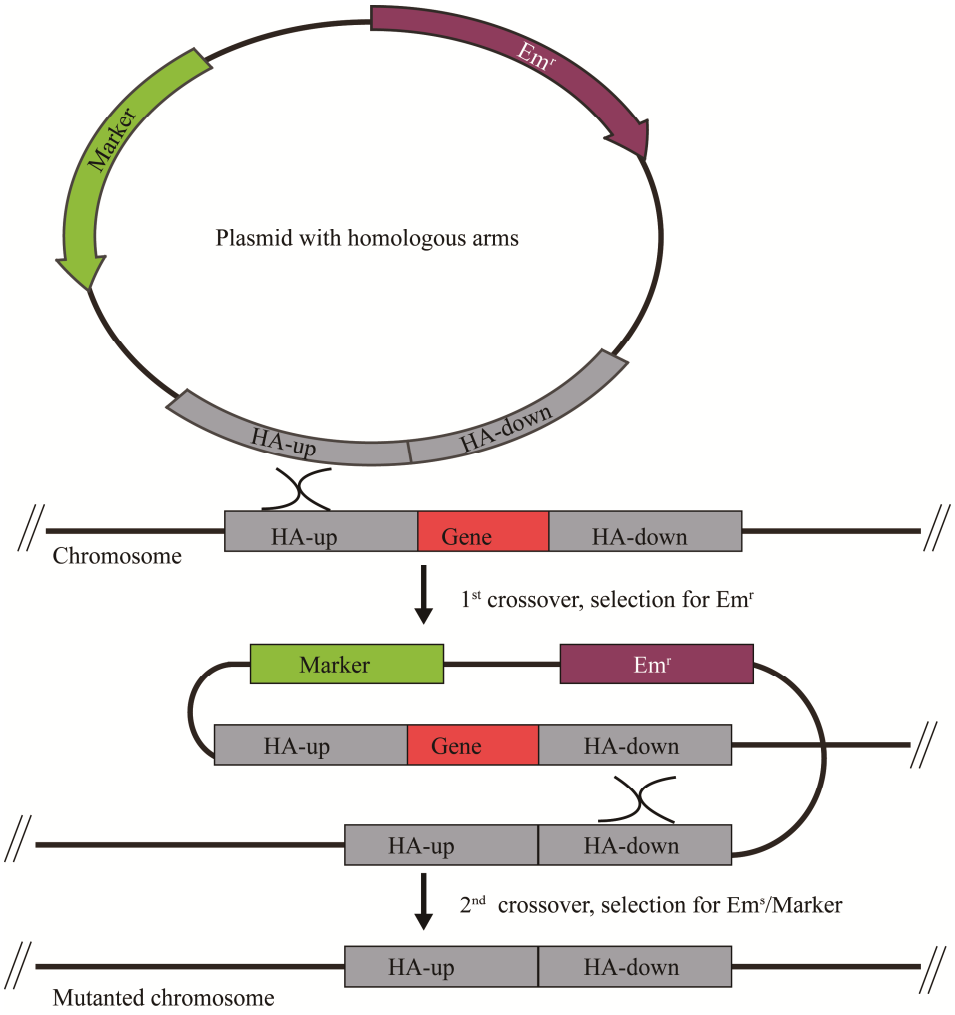

图 1　基于整合质粒的同源重组示意图

Figure 1　Schematic diagram of homologous recombination based on integrative plasmids. HA: Homologous arm. Em^r: Erythromycin resistance cassette.

了基础。由于非复制型整合质粒介导的基因整合对宿主的转化效率要求比较高，随后研究者开发了条件型复制质粒，如热敏性 pG$^+$宿主系统[35]和 RepA 依赖性 pORI 系统[21,43] (衍生于 pWV01)等来提高第 1 轮的交换效率。传统的整合载体筛选标记还是基于双抗性基因分别在 2 次单交换中进行抗性筛选，然后再结合 Cre/*loxP* 位点特异性重组系统对双交换菌株基因组上的抗性标记进行切除，实现基因无标记敲除[19-20]，但会存在 *lox*72 位点残留问题。

1.2　反向筛选系统

为了加快第 2 轮单交换事件中双交换菌株的筛选及质粒去除，反向筛选系统应运而生。例如，广泛应用于谷氨酸棒杆菌(*Corynebacterium glutamicum*) 基于自杀质粒 pK18mobsacB/pK19mobsacB 的基因无痕敲除系统[39,44-45]，其利用果聚糖酶 SacB 作为反向筛选标记，SacB 可以水解蔗糖并合成果聚糖，导致谷氨酸棒杆

菌对蔗糖敏感。许黎明等[46]在 1 株高产 L-乳酸的鼠李糖乳杆菌(*Lactobacillus rhamnosu*, *Lb. rhamnosus*) JCM1553 中引入了基于 pK18mobsacB 的反向筛选系统，成功获得了 *ldhD* 基因缺失的突变菌株，使得发酵液中 L-乳酸含量达到 99.92%。研究表明，编码尿嘧啶磷酸核糖转移酶的 *upp* 基因参与尿嘧啶代谢的替代途径，*upp* 基因的缺失可以赋予突变菌株对 5-氟尿嘧啶的耐药性且不会显著影响宿主的生物学特性[47]。目前 *upp* 基因已作为一种反选择标记在嗜酸乳杆菌(*Lactobacillus acidophilus*, *Lb. acidophilus*)[48]、格氏乳酸杆菌(*Lactobacillus gasseri*, *Lb. gasseri*)[49]、干酪乳杆菌(*Lactobacillus casei*, *Lb. casei*)[50]和 *Lc. lactis*[47]中使用。由于 *upp* 基因在几乎所有的生物体中被发现，因此，该筛选系统的应用需要先构建 *upp* 基因缺失菌株。Solem 等[37]构建了一个含有乳清酸转运蛋白基因(*oroP*)的新载体 pCS1966，用于 *Lc. lactis* 在红霉素或乳清酸作用下进行染色体重组及在 5-氟尿酸盐作用下进行反选择，但是 *oroP* 基因目前并未得到广泛应用。编码苯丙氨酸 tRNA 合成酶 α 亚基的基因 *pheS* 已被证明可以在很多细菌中作为反向筛选标记[51-54]。基于此，Xin 等[38]在 *Lc. lactis* NZ9000 中鉴定了 1 个 PheS 蛋白，证明细胞中 PheS 蛋白发生突变 A312G (PheS*)会使 *Lc. lactis* NZ9000 和 *Lb. casei* BL23 对 p-Cl-Phe (对氯苯丙氨酸)敏感。Zhang 等[55]通过在万古霉素耐药乳酸菌中异源表达二肽连接酶 Ddl 赋予乳酸菌对万古霉素的敏感性，随后将二肽连接酶基因整合到自杀载体上，开发了一种用于万古霉素耐药乳酸菌的反选择标记，从而扩大了目前大多数菌株可利用的基因组编辑工具箱。反向选择标记只允许失去自杀载体的细胞生长，但不能杀死恢复突变(野生型)细胞，因此理论筛选效率只有 50%。

2 基于基因组重组工程的精细修饰和敲除、敲入

基于整合质粒-反筛选系统的基因组整合技术虽然应用广泛，但是整个双交换菌株筛选过程操作烦琐，耗时且低效[20,56]。随着分子生物学和合成生物学技术的发展，一些新兴的基因组改造系统，如基于原噬菌体重组酶的单链 DNA (single-stranded DNA, ssDNA)重组系统及双链 DNA (double-stranded DNA, dsDNA)重组系统成为操作细菌基因组的有力工具[25,57-60]。

2.1 基因组重组工程

自 1998 年以来，通过噬菌体重组酶介导的同源重组使细菌遗传学发生了革命性的变化[61]。其中来源于大肠杆菌 λ 噬菌体的 Red 系统和 Rac 噬菌体的 RecET 系统是最经典的重组系统[62-63]。Red 重组系统由 3 个噬菌体编码蛋白 Redα、Redβ 和 Redγ 组成[64]，RecET 系统仅由 RecE 和 RecT 这 2 个噬菌体编码蛋白组成[63]。蛋白 Redα 和 RecE 作为 dsDNA 依赖性的 5′→3′核酸外切酶，可切除线性 dsDNA 暴露的 3′端 ssDNA；ssDNA-退火蛋白 Redβ 和 RecT 是单链 DNA 退火蛋白(single-stranded DNA annealing protein, SSAP)家族的成员，又称重组酶，可以和 ssDNA 末端结合，保护其不被宿主核酸酶降解的同时促进互补 DNA 链的退火、交换和侵入，这一过程被称为 dsDNA 重组工程[62-63] (图 2A)。然而在 ssDNA 重组工程中只有 SSAP 对促进单链寡核苷酸的重组是必需的[65] (图 2B)。除大肠杆菌外，Red/Rec 系统仅在少数的细菌可以直接使用，可能是由于宿主遗传操作系统及未知重组机制的差异导致[59,66-67]。因此，挖掘内源噬菌体重组酶成为提高重组效率、扩大基于重组工程的基因组编辑技术的有效策略[22,60,68]。

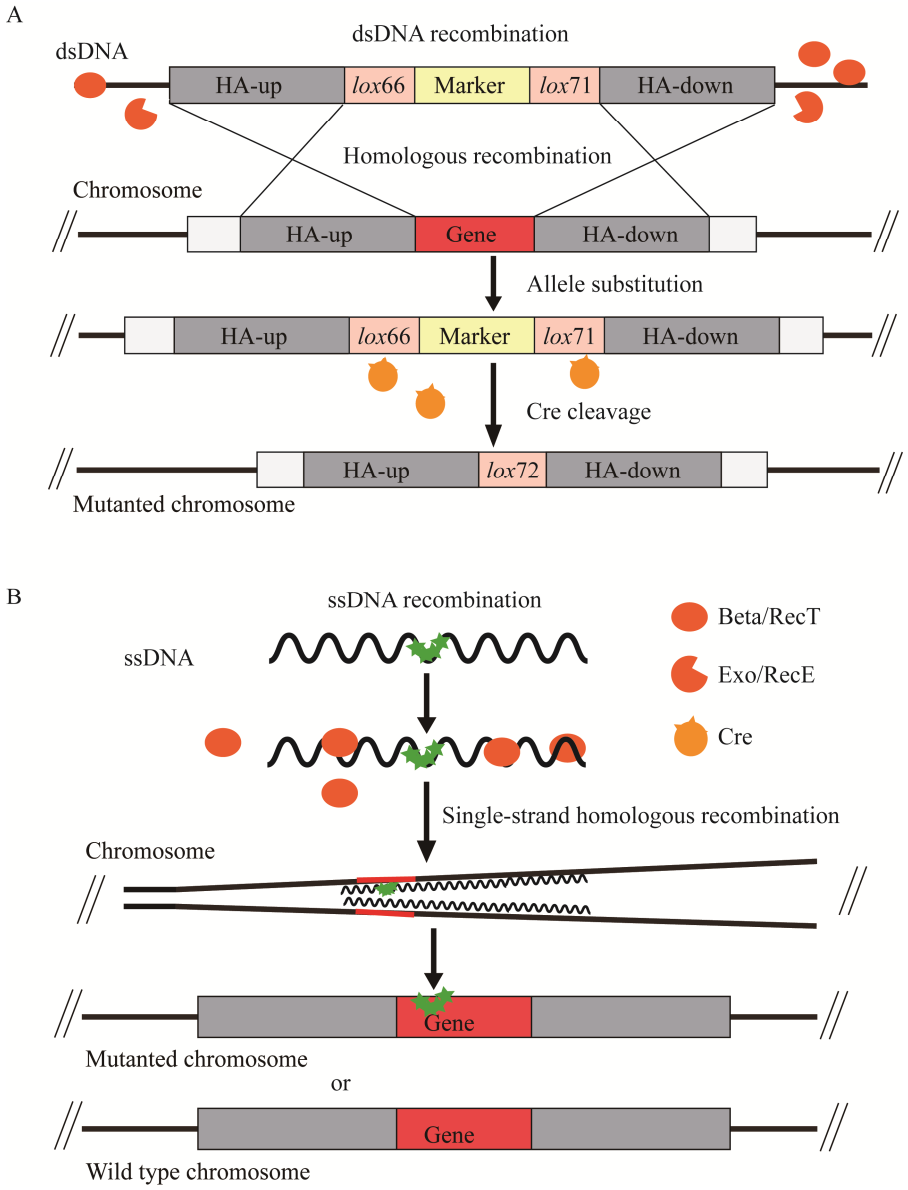

图 2　基因组重组工程原理示意图

Figure 2　Schematic diagram of the principle of genome recombination engineering. A: dsDNA. B: ssDNA. HA: Homologous arm. Beta/RecT: 5′→3′ exonuclease. Exo/RecE: Single-stranded DNA annealing protein. Cre: Cyclization recombination enzyme.

2.2　RecT 介导的 ssDNA 重组

乳酸菌中 ssDNA 重组技术的应用最早是源于 van Pijkeren 等[3]在罗伊氏乳杆菌(*Lactobacillus reuteri*, *Lb*. *reuteri*)中鉴定得到了 ssDNA 结合蛋白 RecT 同系物，在无选择压力下，实现了 *Lb*. *reuteri* 和 *Lc*. *lactis* 基因组精准的碱基突变，但是效率仅达到 0.4%−19%；对重组 *Lb*. *reuteri* 和 *Lc*. *lactis* 菌株的全基因组序列分析表明，ssDNA 重组具有特异性，不具有高致突变性。随后该课题组在乳杆菌和 *Lc*. *Lactis* 中又鉴定

得到一系列ssDNA结合蛋白同系物，同时探索了ssDNA重组系统在*Lb. reuteri*和*Lc. lactis*应用中的优化参数，结果表明，重组酶的来源及表达水平、寡核苷酸的设计和浓度、转化效率及错配修复(mismatch repair, MMR)系统地避免是该系统在乳酸菌中应用的关键因素[2,68]。Guo等[69]通过筛选不同来源的重组酶，研究表明粪肠球菌来源的重组酶RecT在*Lc. lactis*具有最高活性，利用利福平筛选时重组效率可达到100%。ssDNA重组系统的限制因素是在无筛选压力下的效率比较低，导致无表型突变菌株的筛选成为挑战。为了解决这一问题，研究者将ssDNA重组技术耦合高效的酿脓链球菌来源II型CRISPR/Cas9反选择系统，先利用前者使基因组靶标基因和前间隔序列邻近基序(protospacer adjacent motif, PAM)序列发生突变，然后利用Cas9的核酸内切酶活性对未发生突变的细胞基因组进行切割，造成未突变或回复突变菌株的死亡，进一步提高了突变菌株的筛选效率[24,26,69]。

2.3 Red/Rec系统介导的dsDNA重组

Red/Rec系统介导的dsDNA重组大大促进了大肠杆菌及其他几种生物的快速、精确的基因组编辑和功能基因分析[59,67,70-71]。此外，研究表明宿主核酸酶抑制剂Redγ与全长的RecE/RecT或Redα/Redβ表达可有效提高dsDNA和基因组同源区的重组效率[64,72]。基于van Pijkeren等[3]在*Lb. plantarum* WCFS1鉴定的RecT/Redβ同系物Lp_0641，Yang等[23]在*Lb. plantarum* WCFS1原噬菌体P1中鉴定得到潜在的Redγ和Redα蛋白同系物Lp_0640和Lp_0642；随后操纵子Lp_0640-41-42被证明能有效地进行dsDNA底物和宿主基因组DNA之间的同源重组，结合Cre/*loxP*系统，成功在*Lb. plantarum*中开发了一种无标记的基因组操作方法。由于Lp_0640-41-42存在宿主特异性，从而限制了该系统在其他乳酸菌中的应用。Xin等[22]通过生物信息学分析从*Lb. casei* BL23原噬菌体PLE3中鉴定得到1个λRed类似的重组酶操纵子LCABL_13040-50-60，并对它们的功能进行了鉴定；结合Cre/*lox*P系统，在*Lb. casei* BL23中实现了基因的敲除、敲入和精确点突变；此外，在Redγ的辅助下该系统被证明在副干酪乳杆菌(*Lactobacillus paracasei*, *Lb. paracasei*)和*Lb. plantarum* WCFS1中也有重组活性。利用该系统，研究者对*Lb. casei* BL23基因组进行大片段的连续敲除，获得了基因组减少1.68%的突变菌株[73]。dsDNA重组结合Cre/*loxP*系统为乳酸菌作为细胞工厂提供了简单高效的基因组操作工具，但是由于存在*lox*72位点的残留，并不属于无痕敲除。随后，研究者将CRISPR/Cas9反选择系统应用到dsDNA重组系统中实现了目的基因的无痕敲除，并将该无痕基因组操作方法应用到N-乙酰葡萄糖胺代谢途径的改造中[74]。

3 基于CRIPSR/Cas9系统的基因组编辑技术

乳酸菌的遗传多样性导致目前已开发的基因表达元件及基因组操作工具都存在一定的宿主特异性，因此亟须开发一套通用性更强、编辑效率更高、操作更简便的基因组编辑技术。作为细菌和古菌的适应性免疫系统，CRISPR及其相关蛋白(Cas)可以有效地切割进入细胞的外源DNA，如噬菌体DNA或质粒。2013年，Cong等[75]利用CRISPR/Cas9系统在哺乳动物细胞中实现了多基因编辑，使CRISPR技术在生物基因编辑中的应用得到了推广。截至目前，CRISPR/Cas9系统已被广泛用于大肠杆菌[76-77]、

枯草芽孢杆菌[77-78]、谷氨酸棒杆菌[79-80]、酿酒酵母[81-82]等工业重要细胞工厂中的基因组高效、快速的编辑及代谢调控，但是在乳酸菌中仍处于探索阶段。

3.1 CRIPSR/Cas9 系统作用机制

作为天然防御机制，CRISPR/Cas 系统为微生物提供了序列特异性的保护，以抵御外来核酸的入侵[83]。CRISPR/Cas 系统在自然界中极其多样化，并根据其相关的 Cas 蛋白进行分类。其中，II型 CRISPR/Cas 系统(尤其是CRISPR/Cas9系统)由于具有可编程、精确、便携和高效的 Cas9 特征核酸酶的基因组编辑能力，是截至目前研究最广泛的系统[84]。

天然 CRISPR/Cas9 系统由 CRISPR RNA (crRNA)、反式激活 crRNA (trans-activating crRNA, tracrRNA)和 Cas9 核酸酶组成，Cas9 核酸酶与由 crRNA 和 tracrRNA 组成的双 RNA 复合体形成核糖核蛋白(ribonucleoprotein, RNP)复合体，并以 crRNA 的间隔序列识别外源 DNA 的原间隔序列，将 RNP 复合体定位到 PAM 和原间隔序列区域，在PAM上游3个碱基处诱导双链断裂(double-strand break, DSB)[85-86]。然后，在宿主修复途径的帮助下，可以将感兴趣的基因修饰引入突变位点；失去原间隔子或 PAM 序列的突变体可能在 Cas9/tracrRNA-crRNA 复合物的攻击下存活下来[3,24]。后来，研究者将CRIPSR/Cas9系统设计成由Cas9核酸酶和单向导 RNA (single-guide RNA, sgRNA)组成的双组分系统，sgRNA 是一种人工设计的、更简便的 crRNA-tracrRNA 双工形式，包含原有的 gRNA 骨架和可编程的 20 nt 的引导序列，使复合物与引导序列互补到 DNA 靶点[75,87]。因此，基于 CRISPR/Cas9 的基因组编辑可以很容易地通过替换间隔序列来重新编程。

3.2 CRIPSR/Cas9 系统在乳酸菌中的应用

3.2.1 基于 CRIPSR/Cas9 系统的反向筛选工具

由于大多数细菌不存在非同源末端重组(non-homologous end joining, NHEJ)修复机制，当细菌染色体中被引入 DSBs，细胞就会死亡，因此，CRISPR/Cas9 系统可视为一种强大的反向筛选工具来实现预期突变菌株的快速筛选[2,88] (图 3A)。在乳酸菌中，CRIPSR/Cas9 的反向筛选通常是通过耦合基于寡核苷酸的重组工程来实现的，寡核苷酸使 sgRNA 靶向的基因组部位(包含PAM序列)突变，Cas9的序列特异性和致死性只针对未经历重组的细胞[2]。在 *Lb. reuteri* 中，为了提高重组菌株的筛选效率，研究者将CRIPSR/Cas9基因组编辑结合ssDNA重组，使得精确点突变菌株的筛选效率可以达到90%−100%[24]。在 *Lc. lactis* 中，研究者通过选择合适的重组酶和提高 CRIPSR/Cas9 的致死率，可以在 72 h 内实现精确点突变、片段敲除和敲入，筛选效率达到75%以上[69]。在 *Lb. plantarum* 中，Zhou 等[26]通过建立 CRIPSR/Cas9 辅助的 dsDNA 和 ssDNA 重组技术，实现了对基因组的无缝编辑，包括基因敲除、敲入和点突变；随后，他们将这些策略应用于 N-乙酰氨基葡萄糖的代谢工程改造，在不引入外源基因和质粒的情况下重组菌株可产生 797.3 mg/L 的 N-乙酰氨基葡萄糖。此外，CRIPSR/Cas9 系统还可以与整合质粒结合，用于 *Lc. lactis* 中单交换重组菌株的反选择，加快双交换重组菌株的构建和选择[89]。

3.2.2 基于 CRIPSR/Cas9 系统的基因组编辑

除了作为反向筛选工具，CRIPSR/Cas9 系统还被进一步开发应用于乳酸菌基因组和噬菌体基因的编辑技术。*Lc. lactis* 作为一种应用广泛的食品和饮料发酵剂，噬菌体感染一直是食品行业持续存在并引起广泛关注的问题。研究

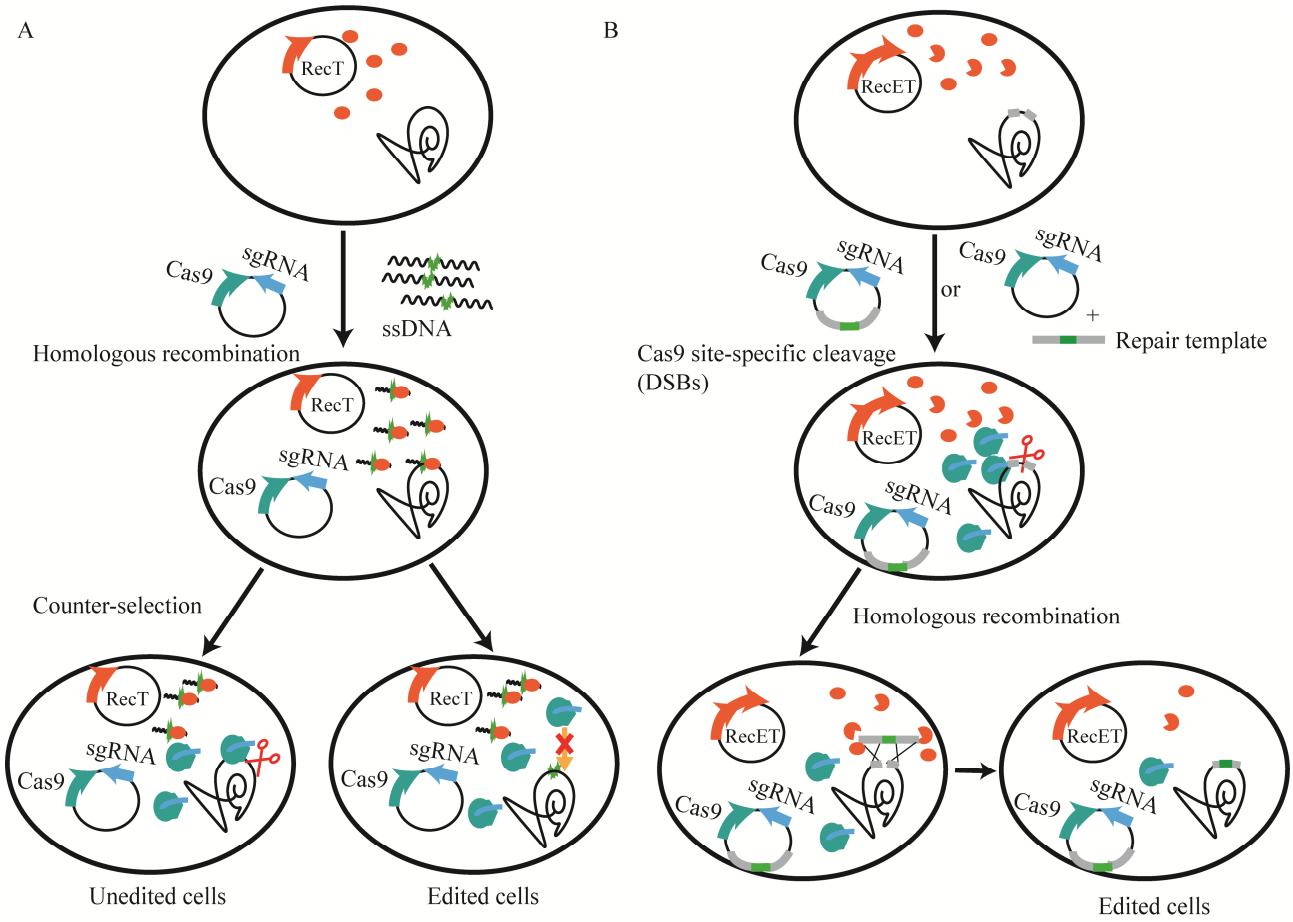

图 3 基于 CRIPSR/Cas9 系统的基因组编辑示意图

Figure 3 Schematic diagram of genome editing based on CRIPSR/Cas9 system. A: Counterselection in precision genome engineering. B: Gene knockout and knock-in of the genome.

者通过对 CRIPSR/Cas9 系统进行优化，成功实现了侵染 *Lc. lactis* 的强毒性噬菌体 p2 的基因组修饰，为研究噬菌体和宿主互作及防御噬菌体感染提供了有效的工具[27]。由于 *Lb. casei* LC2W 较低的同源重组修复(homology directed repair, HDR)潜力，CRIPSR/Cas9 诱导的 DSBs 对 LC2W 是高致死的，而 CRIPSR/Cas9^{D10A}(切口酶)诱导的染色体损伤是一种不太严重的触发修复的方法，因此有助于 LC2W 的基因组编辑[90]。Song 等[29]通过将 Cas9^{D10A} 取代 Cas9，并对 Cas9^{D10A} 和 sgRNA 的表达水平进行优化，在 *Lb. casei* LC2W 中实现了染色体基因的敲除和敲入，效率可以达到 25%−62%；与 CRIPSR/Cas9 介导的 DSBs 相比，切口酶 Cas9^{D10A} 诱导的单链 DNA 断裂(切口)可能更容易修复，从而导致更多的野生型细胞逃逸。随后，他们将内源噬菌体重组酶和 CRIPSR/Cas9 系统相结合，实现了在 *Lb. plantarum* 和短乳杆菌(*Lactobacillus brevis*, *Lb. brevis*)中的基因组编辑，其中单基因敲除在 7 d 内即可完成，敲除效率可达 50%−100%；染色体基因替换效率可达到 35.7%[25] (图 3B)。该研究表明，表达噬菌

体重组酶并引入 dsDNA 修复模板可以有效地提高重组效率，避免 Cas9/sgRNA 诱导的 DSBs 产生的高致死率。这种策略在其他微生物中也已得到验证[91-92]。

3.2.3 基于 CRIPSR/dCas9 系统的基因表达调控和碱基编辑

基因表达调控在研究未知蛋白功能及代谢途径优化方面起到非常重要的作用。除在基因组靶基因处引入点突变、缺失和敲入外，CRISPR 系统还可以通过CRISPR干扰(CRISPR interference, CRISPRi)来调控基因表达[93]。研究者通过构建含 dCas9 和 gRNA 的单质粒系统，在 *Lc. lactis* 中用于 *upp* 基因 mRNA 转录本下调，获得的重组菌株能够在含 5-氟尿嘧啶的半培养基上存活，证实了 *upp* 基因沉默[30]。随后，Xiong 等[31]在 *Lc. lactis* NZ9000 中又构建了用于基因转录抑制的双质粒 CRISPR/dCas9 系统，分别用诱导型启动子 P_{nisin} 和组成型启动子 P44 驱动 SpdCas9 和 gRNA 的表达；结果表明，该系统可以介导单个或多个靶基因沉默，基因表达降低率高达 99%；此外，研究者利用该系统鉴定出假定的青霉素酰化酶 LLNZ_07335 为胆盐水解酶，其对 NZ9000 的胆盐抗性起作用。除了 *Lc. lactis*，研究者也证明了 CRISPR/dCas9 干扰系统可以用于研究 *Lb. plantarum* 中多个重要基因功能[32]，表明 CRISPRi 系统是快速筛选必需基因和非必需基因表型的理想工具。近期，Tian 等[33]将 dCas9 融合胞苷和腺苷脱氨酶分别构建了适用于 *Lc. lactis* 的 CRISPR/cDBE 和 CRISPR/aDBE 碱基编辑器，在无供体 DNA 模板和引入 DSB 的情况下，CRISPR/DBE 可以有效地编辑 *Lc. lactis* 的目标碱基；而且 CRISPR/cDBE 被证明可以在 *Lc. lactis* NZ9000 中同时实现多个基因的失活，其中 2 个基因的失活效率可达 100%。

4 总结与展望

近些年，乳酸菌的安全性和益生作用引起了研究学者的广泛关注[94-95]。此外，许多乳酸菌具有稳健的生理特性，能够耐受乙醇和胃酸等恶劣条件，这使得它们成为工业生产菌株和生物疗法开发的优选载体[15,96]。然而，对于具有工业和医学重要性的微生物菌株，其基因组能否被高效编辑是关键。虽然针对乳酸菌的基因组操作已开发了很多工具和方法，但是普遍存在编辑效率低、耗时长等问题，而且由于乳酸菌的系统发育多样性，多数基因编辑技术存在宿主特异性。因此有必要开发一种简单、高效、通用性更强的基因组编辑技术来促进乳酸菌代谢网络的重构和修饰。

作为新兴的基因组编辑技术，CRIPSR/Cas9 系统仅在少数乳酸菌中实现了基因组编辑，并且 Cas9 核酸酶识别位于靶序列 3′端的富含胞嘧啶的 PAM 序列(5′-NGG-3′)，这也限制了 Cas9 在低 GC 含量乳酸菌中有效的可编辑位点。不同来源或经过改造的 Cas9 突变体具有不同的 PAM 偏好性，根据靶位点选择合适的 Cas9 蛋白，可以拓展该系统在乳酸菌中的可编辑范围[97-98]。V 型 CRISPR/Cas 系统 CRISPR/Cas12 (Cpf1)识别靶序列 5′端富含胸腺嘧啶(T)的 PAM 序列，来源于新凶手弗朗西斯菌的 *Fn*Cpf1 已实现了在多种微生物中的基因编辑，而且研究表明该系统脱靶效率更低，更适合多基因编辑[99-101]。该系统能否适用于乳酸菌基因组编辑有待进一步的开发验证。原核生物 argonaute (pAgo)蛋白是一种新型 DNA 核酸内切酶，可以在 gRNA/gDNA 的引导下实现靶标 RNA/DNA 的切割，目前该系统已经实现了在禽多杀性巴氏杆菌、大肠杆菌中和枯草芽孢杆菌的基因组编辑[102-104]。该系统和 CRISPR/Cas 系统相比无 PAM 序列依赖

性，因此有希望成为新一代基因组编辑技术。

由于菌株遗传背景和生长特性的差异，异源基因编辑技术的开发和应用往往存在转化效率低、酶活性低和细胞毒性的问题。例如，Cas9 核酸酶的表达对很多微生物造成不可避免的细胞毒性，需要通过密码子优化及严格调控 Cas9 蛋白的表达来缓解细胞毒性[79-80]。因此，进一步挖掘或者设计宿主通用强的表达载体和调控元件对实现乳酸菌基因编辑技术的开发至关重要。此外，许多乳酸菌的基因组中含有不同亚型的 CRISPR/Cas 系统，也为基因工程提供了原生的 CRISPR 核酸酶[105-106]。

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