·综 述·

工程微藻的生物安全风险、管控及生物封存

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摘 要：微藻具备利用太阳能固定 CO_2 并转化为有机物的能力，已成为有前途的绿色细胞工厂。随着生物技术快速发展，前沿生物技术在光合微藻中的研究与应用不断拓展，对微藻的工程改造日益全面和深入，比如通过合成生物学和基因组编辑技术对微藻进行工程改造，使其有潜力应用于医学、农业、食品、能源、环境等领域。然而，与此同时，工程微藻在环境中存活和扩散的风险也随之增加，给生态环境和人类健康带来潜在安全风险。为避免其在环境中扩散对生态环境和人体健康造成生物安全风险，需要强化工程微藻生物安全风险管控政策，并针对其开发生物风险防控技术。为了实现这一目标，研究人员开发了生物封存系统(biocontainment)，包括利用有毒蛋白设计杀伤开关等主动策略和敲除必需基因制造营养缺陷型菌株等被动策略，对工程微藻进行空间上的封存。本文对近几年前沿生物技术在微藻生物工程领域的应用、工程微藻逃逸的生物安全风险和管理规范以及工程微藻中建立的多种新型生物封存技术的研究进展进行了总结和评述，最后对微藻生物封存领域的未来发展方向进行了展望。

关键词：工程微藻；前沿生物技术；生物安全；风险管理；生物封存

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Biosafety risks, control, and biocontainment of engineered microalgae

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Abstract: Microalgae, with the ability to harness solar energy to fix CO_2 and convert it into organic compounds, have emerged as promising green cell factories. With the rapid development of cutting-edge biotechnologies, the research and application of photosynthetic microalgae have been expanding, leading to comprehensive and in-depth engineering of microalgae. The synthetic biology and genome editing technologies have enabled the applications of microalgae in medicine, agriculture, food, energy, and the environment. However, the survival and spreading of engineered microalgae in the natural environment pose potential safety risks to ecosystems and human health. To curb the risks caused by the spreading of engineered microalgae in the environment, biosafety policies should be formulated for engineered microalgae and the prevention and control technologies should be developed. Toward this goal, researchers have developed biocontainment systems, including positive strategies such as the design of toxic protein-based kill switches and passive strategies such as knocking out essential genes to construct the strains with nutritional deficiencies, thereby spatially containing engineered microalgae. This article summarizes the application of cutting-edge biotechnologies in the engineering of microalgae, the biosafety risks and management regulations associated with the escape of engineered microalgae, and the progress in novel biocontainment technologies established for engineered microalgae. Finally, this article gives insights into the future development direction of microalgae biocontainment.

Keywords: engineered microalgae; cutting-edge biotechnologies; biosafety; risk management; biocontainment

在化石燃料快速消耗和温室效应加剧的今天，开发及应用更加环保和可再生的能源模式逐渐成为一个重要课题。微藻是能够进行绿色生物合成的重要底盘菌株，但其生产过程需要大量的水、氮、磷和 CO_2[1]，收获和脱水步骤等生产设施和运行费用高[2-3]，导致其生产成本大于化石燃料[4-5]。基因工程改造后的工程藻类提高了藻类的菌株生产力[6]，还在 CO_2 封存和同化[7]及重金属废水处理[8]等方面更具优势；此外，利用其生产的生物燃料逐渐进入大众视野，具有解决工业规模生产经济障碍的潜力，被归类为第四代生物燃料[6]。

微藻中的真核微藻和蓝藻作为藻类基因工程的主要底盘菌株，许多精细化学品和生物燃料已经能够成功地使用工程藻类进行生产[9-10]，除此之外还能够产出如化学前体、动物饲料和微蛋白质等有价值的副产品[11-12]，预计到 2054 年，50%的传统蛋白质将被来自藻类或昆虫的蛋白质取代[13]，但基于微藻的功能性膳食还处于发展的早期阶段[12]。微藻也是第三代生物塑料的原料，基于微藻的生物塑料被认为是传统塑料的替代品[14-15]。微藻的优势在于它能独立进行光合作用，生长周期较短，并且固碳能力强达陆生植物的 10−50 倍[16]，如在平板光生物反应器中，斜针叶藻(*Acutodesmus obliquus*)的 CO_2 固定率可以达到系统总输入 CO_2 的 64%[17]。小球藻属(*Chlorella*)和颤藻属(*Oscillatoria*)中的代表已经显示出良好的 CO_2 捕获能力[18-19]。其中小球藻(*Chlorella vulgaris*)在含 CO_2 吸附剂的微藻反应器中培养时，还能提高 CO_2 固定效率[20]。研究人员评估微藻理论上的 CO_2 固存性能，在 10 万 km²养殖面积中，微藻的最大固碳潜力为 2.35 Gt CO_2，平均每年约为 3.243 3 亿 t[21]，Iglina 等[22]也报道，在 4 000 m^3 体积的光照的池塘中培养藻类，每年 CO_2 吸收量约为 2 200 t。这些例子凸显了微藻的环境效益[23]。与此同时，微藻的适应能力很强，可以在户外或者开放的池塘中生长，甚至利用盐水或废水生长，不占用农业用地[6,24]。基于微藻的 CO_2 固存能力，以 0.750 g/(L·d)的速度，一年可从废水中固存最多 273.75 g/L 的 CO_2[25]。在生长过程中，微藻将废水中存在的营养物质纳入其生物质中，进一步生产如生物燃料、生物炭和类胡萝卜素等高价值产品[26]，或与其他微生物相互作用，提高产品收获量的同时也能够处理废水中的有机物和有毒物质如重金属离子等[27-29]。近年来多个国家和地区都开始重视光合微生物用于新型生物能源的合成生物学研究，自 2010 年以来，美国能源部等机构已经陆续投入数亿美元，来支持采用合成生物学方法对光合微生物进行改造，生产绿色高效能源及进行化学品的研究与开发[30-32]。我国也同样重视对清洁能源的开发和光合微生物合成生物学的研究，“十三五”的“合成生物学”重点研发专项中将光合蓝细菌的合成生物学研究列为一个重要内容。在微藻中能够通过基因组编辑的方法，如 CRISPR 技术敲除磷脂酶[33]或抑制磷酸烯醇丙酮酸羧化酶[34]等基因来增加脂质的积累[35]，采用 RNA 干扰(RNA interference, RNAi)技术敲低丙酮酸脱氢酶激酶的表达也能将三角褐藻(*Phaeodactylum tricornutum*)的中性脂质由 23.1%增加到 42.1%[36]。由此可见，利用前沿生物技术改造的工程微藻成为世界范围的重要底盘。

利用合成生物学、基因组编辑等前沿生物技术对微藻进行系统改造，拓宽了其进一步工业化应用的可能性。但与其他转基因生物一样，微藻大规模种植之前必须评估其可能带来的生物安全风险，并采取适当措施来降低这些风险。Henley 等[37]对基因工程微藻进行了风险评估，发现通常情况下其对人类健康、环境和经济的风险很低，但也并非为零。考虑到基因工程藻类可能在户外及开放的池塘中生长，这些工程细胞逃逸到环境中的可能性确实比其他典型的工业微生物培养方式更高。因此，Henley 等建议开发生物防护策略，降低工程藻类在自然环境中的生长适应性，当细胞离开实验室或工业环境时，对它们进行有条件的杀伤，并降低其将遗传物质转移到其他生物体的能力[38-39]。光合蓝细菌作为“自养型细胞工厂”应用时，往往采取室外培养的方式，这使得其菌体或 DNA 进入环境的几率加大，而经过工程改造的微藻携带新型的遗传元件和模块，进入环境可能带来不可预测的后果[38]。

此外，具备某种竞争优势的合成生物进入环境，通过与本地物种竞争或者 DNA 重组，可能会对生物多样性产生影响，甚至可能和其他土著种微生物发生重组，增加造成生物安全风险的几率[40]。研究人员利用前沿生物技术研发出多样的工具与方法，显示出了在改造微藻生产生物燃料上的巨大潜力[41-44]，然而环境风险成为了限制工程微藻工业生产的主要因素[3]。

生物封存(biocontainment)是指建立限制重组 DNA 传播的生物屏障，能够通过设计防止工程微生物在指定环境如实验室和限定生产环境等以外生长繁殖或释放体内基因[38,45]。生物封存技术常被分为被动和主动两种形式，被动形式主要是通过缺失必需基因[46-48]建立营养缺陷型菌株。最近报道的硫胺素营养缺陷型布拉氏酵母菌(*Saccharomyces boulardii*)，在实验室生长条件下和小鼠胃肠道中均进行了验证，为微生物组疗法提供了一种可能的生物防护菌株[49]。主动形式通常使用毒素/抗毒素(toxins/antitoxins, TAs)基因[50]和自杀开关[51]。如 Rottinghaus 等[52]在大肠杆菌(*Escherichia coli*)中开发的基于 CRISPR 的化学响应以及化学温度双响应杀伤开关，均可以使 *E. coli* 在小鼠肠道内被有效地选择性杀死，展现了强大的生物防护能力。由此可见，*E. coli* 等细菌底盘中的生物防护技术已经有所发展。Zhou 等[53]在蓝细菌中进行了自杀开关的相关研究，证明了整合的铁离子诱导启动子 PisiAB 和 TAs 可以成为蓝藻生物封存的有用工具，该系统在 3 d 后逃逸频率低于检测限 10^{-9}/CFU，成功构建了聚球菌的第一个活性生物遏制系统。

工程化藻类在开放型系统中进行规模化生产，更应充分注重其可能出现的风险，通过技术手段开发和建立生物遏制策略，避免或减轻其生物安全风险，促进微藻工程的绿色健康发展。因此，本文将对前沿生物技术在微藻生物工程领域的应用、工程微藻逃逸的生物安全风险及管理规范以及工程微藻中建立的多种新型生物封存技术的研究进展进行总结和评述。同时，对微藻生物封存领域的未来发展方向进行展望，为工程化微藻的进一步应用提供借鉴与参考。

1 前沿生物技术在微藻中的应用现状

近年来，生物科技领域发展迅猛，以合成生物学、基因组编辑、基因驱动为代表的前沿生物技术不断取得突破性进展，这些技术逐步应用于农业食品、生物资源与生物安全等领域。生物科技的发展深刻地改变了人类对生命本质及其运行规律的认识，引领了医学、农业、食品、能源、环境等领域的发展[54]。将前沿生物技术与微藻相结合，能够逐步加深对藻类生物学的理解，构建在未来挑战中更有适应性的新型生产菌株，推动基于微藻的生物经济的建立。

1.1 利用合成生物学技术对微藻的改造

合成生物学融合了化学、生物学、工程学和信息技术等学科[55]，能够利用最基本生物元件对生物系统进行修改和重新组装，构建符合预期功能的生物体，实现生物技术应用中生物内部代谢配置的优化[56]。基因组测序领域的发展，进一步促进了微藻这一具有前景的微生物底盘中遗传工具的开发[57]，使微藻合成生物学研究取得了重大进展。遗传元件，如内源性启动子/终止子对[58]、优化的启动子[59-60]、核糖开关[61-62]以及筛选标签、报告基因和蛋白质标签[63-64]等，均在多种微藻中被表征并开发为调节基因表达的工具。这些工具对于靶向多个位点、引入多基因途径或构建独立合成电路等研究都至关重要[65]，推进了微藻生理遗传学基础和微藻代谢工程的研究。在微藻生物燃料生产方面，常用的基因工

程策略包括过表达参与生物质合成的酶等相关基因[66-68]，切断竞争代谢途径[67,69]，或利用转录因子调节生物合成途径[70]，这些方法已经应用于微藻中提高底盘淀粉、脂质以及其他生物燃料产量的研究。如在集胞藻(*Synechocystis* sp.) PCC 6803 中敲除酰基-ACP 合成酶基因，将生物酒精的产量提高了约 26.0 倍[71]，在两种小球藻中表达碳酸酐酶使脂质的积累量提高了约 2.2 倍[72]。微藻作为制药平台生产重组药物可以通过建立起的叶绿体或细胞核表达途径实现。叶绿体表达途径的一些有效元件已被开发，如带有特定启动子和 5′非翻译区的优化载体[73]。到目前为止，几种微藻来源的生物药物已在临床前水平进行了评估，其中利用莱茵衣藻(*Chlamydomonas reinhardtii*)叶绿体表达的人用候选疫苗[74-75]就是备受关注的一种。综上，合成生物学技术对微藻底盘改造领域有着显著的推进作用。

1.2 基因组编辑技术在微藻中的应用

生命系统的基因组几乎能够编码所有功能的指令[76]，近年来发展的基因组编辑技术使定向操纵所有类型细胞或生物体的基因组有了实现的可能[77]。基因组编辑技术有助于实现微藻的基因组改造，是微藻底盘改造的另一个有效策略[78]。基于核酸酶的技术主要有：锌指核酸酶(zinc-finger nucleases, ZFNs)、转录激活因子样效应核酸酶(transcription activator-like effector nucleases, TALEN)和 CRISPR 系统[77]。其中 CRISPR/Cas 系统是古细菌和细菌中的适应性噬菌体免疫系统[79]，已成为基因组编辑领域的最新平台，广泛应用于微藻底盘的优化细胞代谢、调节生物合成途径、提高代谢物的速率和产量[80-81]。首个用于 *C. reinhardtii* 基因编辑的 CRISPR/Cas9 技术在 2014 年被报道，Jiang 等[82]在 *C. reinhardtii* 中证明了 Cas9 和 sgRNA 基因正常发挥作用，能够导致靶向基因修饰。不久后，Ajjawi 等[70]利用 CRISPR 诱导敲除或减弱调控基因增加了工业微拟球藻中脂质积累。利用 Cas9 或 Cpf1 的基因组编辑也已成功应用于不同种类的蓝藻[83]。Wendt 等[84]利用 CRISPR/Cas9 系统能够在蓝藻(*Synechococcus elongatus*) UTEX 2973 中实现无痕基因组修饰。随着 CRISPR 技术的发展，在微藻底盘中插入靶基因的方法也逐渐被建立。目前有 3 种方法用于在微藻底盘中插入靶基因，包括基于整合质粒的基因组编辑、基于复制质粒的基因组编辑以及基于核糖核酸蛋白(ribonucleoprotein, RNP)的基因组编辑。其中 RNP 诱导的基因组编辑已经在 *C. reinhardtii*[85]、胶球藻(*Coccomyxa* sp.)[86]中成功实现靶基因的插入。利用多个 RNP 复合体，实现了 *P. tricornutum* 中多个基因的同时敲除[87-88]。RNA 干扰(RNA interference, RNAi)通过阻碍特定基因的转录或翻译来抑制基因表达[89]，其介导的基因沉默途径已在 *C. reinhardtii* 中进行了研究[90-91]，可能发展成为微藻中靶向基因敲除的有效工具[92-93]。

1.3 其他前沿生物技术在微藻中的应用

微藻生产生物燃料具有可观的商业前景，但目前相比于石油产品，其成本竞争力还很弱[94]。代谢工程有助于提高微藻生产力、降低成本，但微藻中确切的代谢途径和众多相关基因的作用仍是未知的。生物信息学在微藻基因的注释[95]、蛋白质的细胞定位[96-97]和预测藻类结构及蛋白质相互作用数据[98]方面都提供了相应的帮助。一旦有了注释的藻类基因组或转录组，就可以建立相应的藻类代谢模型如 iRC1080[99]和 AlgaGEM[100]，并分析藻类物种的代谢网络拓扑结构[94]。各种学科的交叉应用以及新技术的飞速发展使研究人员对微藻的理解逐步加深，对其改造的程度也越深越广。目前工程微藻还处于起步阶段，大多数底盘在实验室条件下运转很好，但工程微藻扩大培养的可行性仍待提高，尤其针对环境和经济方面。真正实现工程

微藻的工业化，仍需要研究人员更多的关注和努力[101]。

2 工程微藻环境逃逸的生物安全风险及管理规范

利用前沿生物技术来改造微藻底盘的性状，能够提高微藻的应用潜力，但同时，与其他工程菌株一样，工程微藻逃逸至自然界会给生态环境和人体健康带来潜在风险。

2.1 工程微藻逃逸引发生态环境风险

环境中生物入侵通常分为扩散、建立和持续3个阶段[102]，这种入侵可能会改变本地的生态特性，如群落中的优势物种和生态系统的物理特征等[103]。藻类生物燃料可以在封闭的光生物反应器中培养，但仍可能会意外释放，如生物反应器破损或排除废弃培养基时没有彻底灭菌导致的泄漏[104]。更值得关注的是，工程微藻的大规模培养通常是在开放的环境中如池塘、海洋附近或海水中进行[105]。另外，藻类能够自然地适应水体和土壤生态系统的广泛变化，可以轻易通过非生物(风和雨)和生物方式传播，这进一步加深其逃逸引发的生态风险[106]。尽管目前有学者认为，多数微藻商业用途的基因性状在自然界中不太可能具有竞争优势[37]。如Flynn等[107]通过模拟分析认为，逃逸的工程微藻种群可能很快就会被自然种群淘汰。但也有学者认为，工程微藻会形成有害的藻华，还可能产生藻毒素，影响生态平衡[108]。

2.2 工程微藻逃逸造成公共健康风险

有害藻华的形成不仅会对生态健康产生影响，也可能会危害到人类的生命安全，导致人类中毒风险升高[109]。比较安全的微藻遗传操作的基因工程技术包括自我克隆和诱变，这两种方法产生的微藻基因组中不含有外源DNA序列[110]，但为了应对第三代生物燃料面临的经济障碍，必须开发和利用各种基因技术，从而能够广泛地对微藻进行基因改造[111]。染色体或质粒DNA的扩散可引起微生物的水平基因转移。即使工程微藻自然环境适应性上不太具备竞争力，其体内的DNA在生物体死亡后仍能够存在一定的时间[112]，仍具有水平转移的风险[113]。抗生素耐受基因的转移可能导致天然细菌获得抗生素耐药性，一些基因片段也可能流入当地农作物中，对人类健康造成不可预估的危害[114]。工程藻类的扩散导致的潜在公共健康风险仍然是一个重大问题[82-84]，有预见地对工程微藻进行评估和监管，采用物理或生物手段防止工程微藻逃逸成为重要的研究方向。

2.3 工程微藻户外培养试验及管理规范

工程微藻逃逸可能产生的潜在后果表明，工程微藻的工业化进程需要在政府法规及转基因生物监管机构的严格督导下实施，保护社会稳定、人类健康以及环境安全[115-116]。在美国，负责监管的机构主要有美国食品药品监督管理局(Food and Drug Administration, FDA)、美国农业部(United States Department of Agriculture, USDA)和美国环境保护署(U.S Environmental Protection Agency, EPA)等。欧盟委员会建立了转基因生物法以确保现代生物技术(尤其是转基因生物)的开发在安全条件下进行，对包括工程微藻的所有转基因生物的相关风险问题做出了相应规定[117]。墨西哥于2000年成立生物安全和转基因生物部际委员会，负责协调墨西哥联邦公共行政政策，涉及生物安全和转基因生物的生产运输、进出口、繁殖和消费[118]。墨西哥还于2005年发布了《生物安全法》[119]，该法规是比较全面的生物技术法规。印度于2011年出台的《机构生物安全委员会指南和安全手册》中说明了生物遏制措施的有关内容，其中的一个方法是采取生物加物理协同控制的措施，以此来克服单独采用物理控制措施可能产生的困难和不足[120]。印度政府在2013年提出了一项印度生物技术监管局法案，该法案将允许印度科学家在没有任何政

治干预的情况下进行转基因研究[121]。在我国，主要由农业农村部负责管理农业转基因生物。《农业转基因生物安全评价管理办法》于2002年1月5日以我国农业农村部令第8号发布，2022年1月21日以农业农村部令第2号修订[122]，其中第十六条的内容包括“农业部依法受理农业转基因生物安全评价申请。申请被受理的，应当交由国家农业转基因生物安全委员会进行安全评价。国家农业转基因生物安全委员会每年至少开展两次农业转基因生物安全评审。中华人民共和国农业农村部收到安全评价结果后按照《中华人民共和国行政许可法》和《条例》的规定作出批复。”

目前也有基于政府指导的工程微藻工业化的案例。2010年美国能源部(United States Department of Energy, USDOE)授予蓝宝石能源公司(Sapphire Energy Inc.) 5 000万美元，用于建设和运营新墨西哥州哥伦布市的一个用藻类农场生产可再生生物原油的示范规模项目[123]。该公司还在2013年8月1日提交了一份综合有毒物质报告物质控制法案(toxic substances control act, TSCA)的环境释放申请(TSCA environmental release application, TERA)并于2013年9月25日获得批准进行现场测试并缩小规模进行户外实验[124]。2010年USDOE给Algenol等拨款2 500万美元，用于进行一个通过光合作用将太阳能转化为乙醇的综合试点项目，并研发一套可用于商业的光生物反应器系统[125]。美国农业部动植物卫生检验局生物技术监管部门判定他们使用的菌株对植物、动物或人类均无致病性，因此不受植物保护法的监管。2014年，此直接制乙醇途径获得了EPA的批准，同时由于Algenol提议在户外采用封闭的光生物反应器，EPA也表示该设施将免除TERA申请流程。González-Morales等[126]用磷选择性系统控制室外培养蓝藻过程中的生物污染，遵守墨西哥的生物安全法规，采用加入BG-11培养基的自来水捕捉并监测转基因菌株在环境中的潜在扩散。

3 工程微藻生物封存研究进展

工程生物体在工业、临床和环境保护等领域的应用越来越多，这也使危险生物实体传播到环境中的风险变得越来越大。为了解决这一生物安全问题，创建了限制这些生物和转基因材料的方法。合成生物学、基因组编辑等新兴技术的出现，推动了新型生物防护系统的发展[38]，现有的不同的生物防护系统各自具有其优缺点，在防止工程生物体传播的过程中有不同的侧重(表1)。

3.1 工程微藻DNA层面的封存技术

遗传信息的流向通常遵循中心法则，阻断DNA的复制可以截断遗传信息的扩增，因此研究人员希望构建天然核苷酸合成酶缺陷型微生物或非天然核苷酸合成缺陷型菌株实现封存(图1)。如Steidler等[46]用人合成白细胞介素-10 (interleukin-10, IL-10)基因替换乳酸乳球菌胸腺苷酸合成酶基因 *thyA*，得到依赖于外源胸腺添加的营养缺陷株。采取这种方法，成功建立起一种“基因防火墙”，这种防火墙能有效防止转基因生物与自然生物之间基因的水平转移[127]。Luo等[128]利用新兴的异种核酸(xeno-nucleic acids, XNAs)家族也能达到相同的目的。核酸酶蛋白如非特异性核酸内切酶(NucA)[129]、限制性内切酶*Eco*R I等以及其他核酸酶如CRISPR/Cas系统也可以降解DNA使细胞死亡，在工程菌中已经有了相关生物封存技术的应用[51,130-131]。此外，直接敲除基因来降低竞争适应性或产生代谢缺陷的遏制方法也被广泛使用，如来自实验室的*E. coli* K-12菌株常含有降低饥饿情况存活率的*relA*突变[132]。此外，还可以将微藻设计成依赖于实验室特定条件或添加特定化学物质才能维持生长的营养缺陷型菌株。如CO_2，其在自然水体中的

表 1　生物防护系统

Table 1　Biological protection system

Stage of the central dogma	Containment system	Advantages	Disadvantages
DNA level	Construction of natural nucleotide synthase auxotrophic	(1) Easy to implement (2) Resist mutation escape	The required natural metabolites might be obtained in the natural environment
	Knockout essential genes	(1) Easy to obtain (2) Resistant against mutational escape	Key essential genes require large-scale screening
	Unnatural base pairs or xeno nucleic acids	(1) Highly resistant to mutation escape (2) Natural organisms cannot replicate or transcribe any released gene fragments, which can prevent horizontal gene transfer	Difficult to develop and apply
Transcription level	Using synthetic gene circuits	(1) Numerous available effectors (2) The containment system might be universality	(1) Prone to develop mutant escape strains (2) Signal crosstalk may occur, leading to system deactivation
Translation level	Construction of natural amino acid auxotrophic	Resistant to escape caused by key gene mutations	Possible acquisition of required natural metabolites from the natural environment
	Non-canonical amino acids auxotrophic	(1) Strongly resistant to mutation escape (2) It is possible for all kinds of natural organisms	Difficult to develop and apply

丰度较低，藻类因此进化出了 CO_2 浓缩机制(CO_2 concentration mechanism, CCM)，敲除羧基壳蛋白或无机碳转运体的基因会破坏这一机制，从而要求有一个高浓度的 CO_2 环境来维持正常生长。Clark 等[133]构建缺乏羧基壳蛋白的模型蓝细菌聚球藻(*Synechococcus*) PCC 7002，其在空气中存活率少于 5×10^{-10}/CFU，低于美国国立卫生研究院(National Institutes of Health, NIH)建议的 1×10^{-8}/CFU。磷(P)是所有生物体必需的元素，磷酸盐(H_3PO_4, Pi)及其酯是在自然环境中大多数生物可利用的形式，只有个别的细菌群体可以代谢如亚磷酸盐(H_3PO_3, Pt)和次磷酸盐(H_3PO_2, HPt)等其他磷化合物。Motomura 等[134]将这种 Pt/HPt 依赖性机制引入到蓝细菌中，创造了亚磷酸盐营养缺陷型的蓝藻菌株：Pt 同化能力通过表达 Pt 脱氢酶(Pt dehydrogenase, PtxD)和次磷酸盐转运蛋白基因获得，这些基因允许蓝细菌聚球藻(*Synechococcus elongatus*) PCC 7942 吸收 Pt 而非 Pi；然后敲除两个内源 Pi 转运蛋白 *pst* 和 *pit* 基因，所得菌株无法在含有除 Pt 之外的各种类型 P 化合物的任何培养基上生长，且至少 28 d 没有产生任何逃逸突变体，检测限为 3.6×10^{-11}/CFU。除了蓝细菌，亚磷酸盐介导的选择和培养也已应用于其他微藻如 *C. reinhardtii*[135]和新兴模式藻类海洋微藻雷诺氯单胞菌(*Picochlorum renovo*)中[136-138]。Slattery 等[139]使用 CRISPR/Cas9 成功编辑了 *P. tricornutum* 尿嘧啶、组氨酸和色氨酸生物合成途径中的基因，扩大了基因组工程应用的范围，并为工程 *P. tricornutum* 菌株的生物控制提供了一种手段。破坏硝酸还原酶基因构建营养缺陷型菌株，也作为一种措施在多种微绿藻属如微拟球藻(*Nannochloropsis gaditana*)和海洋微拟球藻(*Nannochloropsis oceanica*)[70,140]中以及 *P. tricornutum* 中[141-142]进

行了研究。如前文所述，DNA 水平的生物封存系统多数包含营养缺陷型菌株的建立(表 2)。

DNA 水平的封存还可以基于 DNA 水平的细胞降解来实现。如 Čelešnik 等[104]利用使用金属离子诱导启动子表达来自鱼腥藻(*Anabaena* sp.) PCC 7120 的非特异性 DNA/RNA 核酸酶 NucA 及其抑制剂 NuiA，在诱导剂存在的情况下造成了有效的细胞杀伤。这种条件致死依赖于 DNA 的细胞内降解，既能实现对细胞杀伤，还能破坏遗传物质，降低基因水平转移的风险。

3.2 工程微藻转录层面的封存技术

在 RNA 转录水平上的生物封存常采用设计合成基因电路的技术，合成电路中广泛使用化学诱导型启动子来实现条件致死的效果，一种方法是通过诱导性启动子控制 TAs 系统，在转录水平上根据培养条件调整毒素和抗毒素含量，使工程菌株在非培养环境中缺乏抗毒素而死亡[50,143](图 2)，目前多种 TAs 被开发用于生物遏制[50,144-145]。Čelešnik 等[104]用组成型启动子 PrnpB 控制来源于 *Synechocystis* 6803 的 *slr0664* 毒素，用铜反应启动子 PcopB 控制其对应的抗毒素；构建的菌株在标准 BG-11 中比野生型生长减少，而在补充 4 μmol/L 锌离子的 BG-11 中与野生型生长相仿。尽管 TA 系统是抑菌而不是杀菌作用，但这种杀灭开关也可以作为藻类遗传工程的反选择标签。Zhou 等[53]筛选了几种金属离子诱导型启动子，从中鉴定出一个渗漏率较低的铁离子抑制启动子 PisiAB，并测试了 4 个蓝细菌内源性的 TA 系统 *ssr1114/slr0664*、*slr6101/slr6100*、*sepA1/sepT1* 和 *sepA2/sepT2*，使用中等强度组成型启动子 PpsbA2 表达相应的抗毒素，构建出了 *S. elongatus* PCC 7942 的第一个活性生物遏制系统；其中 *sepA2/sepT2* 符合严格有效的生物遏制系统的标准，系统构建的转基因菌株逃逸频率低于 10^{-9}/CFU[53]。另一种转录水平的封存方法是利用诱导性启动子和相应的信号分子控制必需基因的表达，Hoffmann 等[146]筛选酵母基因库选择了 3 个基因(*SPC110*、*DIS3* 和 *RRP46*)作为合

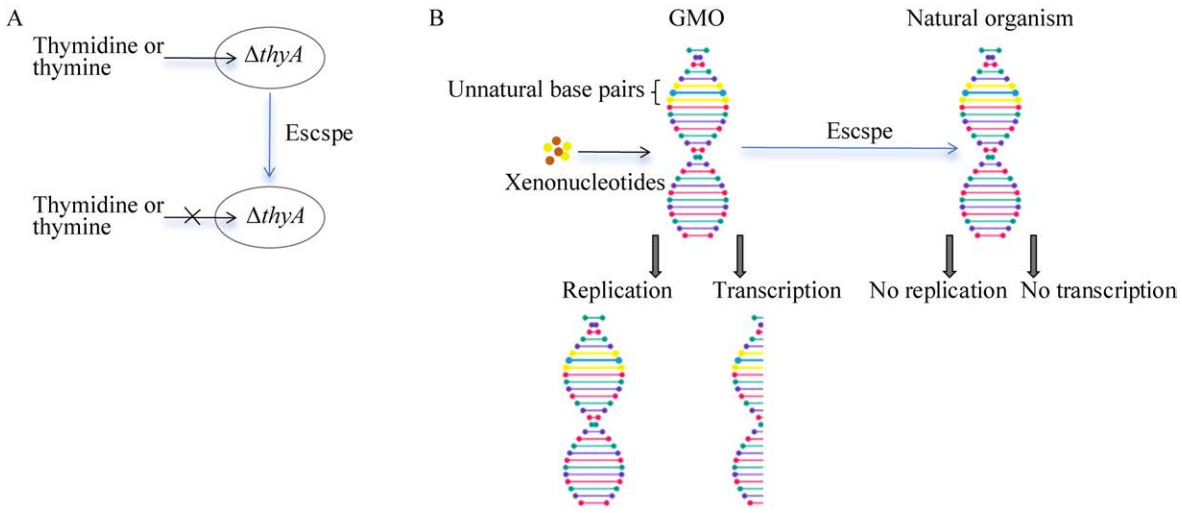

图 1 基因复制层面生物封存的两个例子

Figure 1 Two examples of biocontainment in the level of gene replication. A: In *Streptococcus lactis*, the deletion of the *thy*A gene renders it capable of growing only in an environment with exogenous thymidine supplementation. B: Genes containing heterologous nucleotides can be replicated or transcribed in the engineered host (left side), but not in natural organisms (right side).

表 2　营养缺陷型菌株类型

Table 2　Types of auxotrophic strains

Description	Permissive condition	Host microorganisms	Effectiveness	References
Knock out *thyA*	Exogenous thymidine or thymine addition	*Lactococcus lactis*	When deprived of thymidine or thymine, the viability of strain would drop and prevent its accumulation in the environment	[46]
Mutation of *relA*	Express the *relA* gene	*Escherichia coli*	Without *relA*, bacteria could not survive in a nature ecosystems, because they are unable to synthesize ppGpp during amino acid starvation	[132]
Delete carboxysome shell protein	High CO_2 concentration (>5%)	*Synechococcus* PCC 7002	The abundance of carbon dioxide in the surrounding environment is too low for the strains to grow normally	[133]
Disrupted two indigenous Pi transporters	Introduce Pt dehydrogenase (PtxD) and hypophosphite transporter (HtxBCDE) genes	*S. elongatus* PCC 7942, *C. reinhardtii*, *P. renovo* and *P. celeri*	The strain can not use Pi and would rapidly lose viability in the absence of Pt	[134-138]
Use Cas9 to knockout the *PtUMPS* gene and the *PtAPT* gene to create adenine auxotrophs	Plasmid-based copies of the intact *PtUMPS* and *PtPRA-PH*/*CH* genes can complement the uracil- and histidine-requiring phenotypes	*P. tricornutum*	The knockout strains can not survive in the absence of uracil and histidine in natural environment	[139]
Nitrate reductase deficient strains	Ammonium supplementation or gene complementarity	*N. gaditana*, *N. oceanica* and *P. tricornutum*	The strain in nitrogen-starved state can not survive in the culture medium with nitrate as the sole nitrogen source	[70,140-142]

适的靶标。利用雌二醇的存在与否控制菌株的生长。Ishikawa 等[147]从不动杆菌(*Acinetobacter* sp.)基因组删除了编码必需外膜蛋白的 *bamA* 基因，用表达了受 Pu 启动子控制和调节蛋白 XylR 调节的 *bamA* 基因质粒进行回补，达到了减少逃逸突变体的目的。

3.3　工程微藻翻译层面的封存技术

翻译的过程中氨基酰-tRNA 合成酶(aminoacyl-tRNA synthetase, aaRS)会与其相应的 tRNA 结合。通过在菌株中引入一个非正规 aaRS，并将遗传密码子重新分配给非天然氨基酸(non-canonical amino acids, ncAAs)，使蛋白质的翻译依赖于添加的 ncAAs，构建开发 ncAAs 营养缺陷型菌株，可实现菌株的封存。通常 ncAAs 被分配为宿主不偏好的终止密码子用以合成必需基因来控制细胞生长[148-149](图 3A)，或将有义密码子重新分配给具有完全不同化学特征的氨基酸密码子，如将标准亮氨酸 CUG 密码子解码为丝氨酸[150](图 3B)。在 *C. reinhardtii* 的叶绿体中，没有一个蛋白基因使用了终止密码子 UGA，因此其可以作为潜在的合成生物学工具，经过重新分配给正义密码子而产生翻译屏障。Young 等[151]将 *E. coli* 胞嘧啶脱氨酶基因 *crCD* 的多个 TGG 密码子修饰为 TGA 密码子，防止基因在 *E. coli* 和叶绿体中的功能性表达，随后在 *C. reinhardtii* 中利用合成的 tRNA 将 TGA 密码子改读为色氨

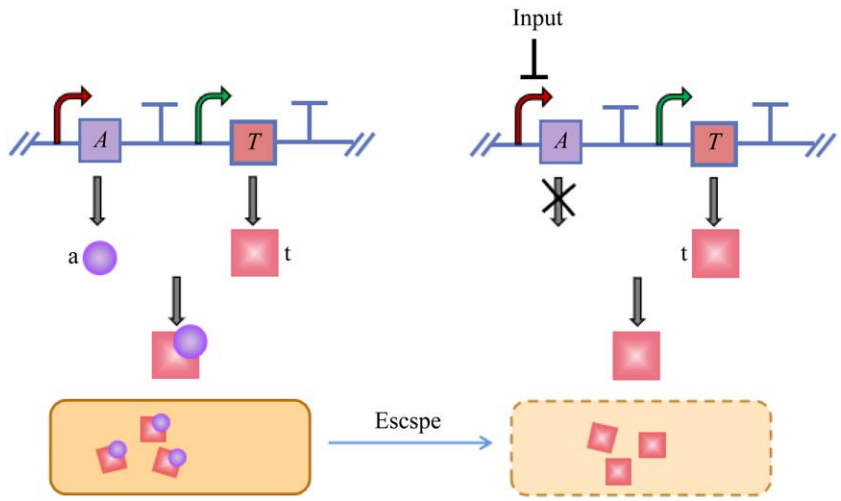

图 2　使用毒素对的基因电路

Figure 2　Genetic circuits with toxin pairs. After the engineered strain escapes, the activation conditions for the antitoxin (*A*) gene promoter are no longer present. Consequently, the accumulation of the toxin (*T*) results in the demise of the engineered strain. a: Antitoxin protein; t: Toxin protein.

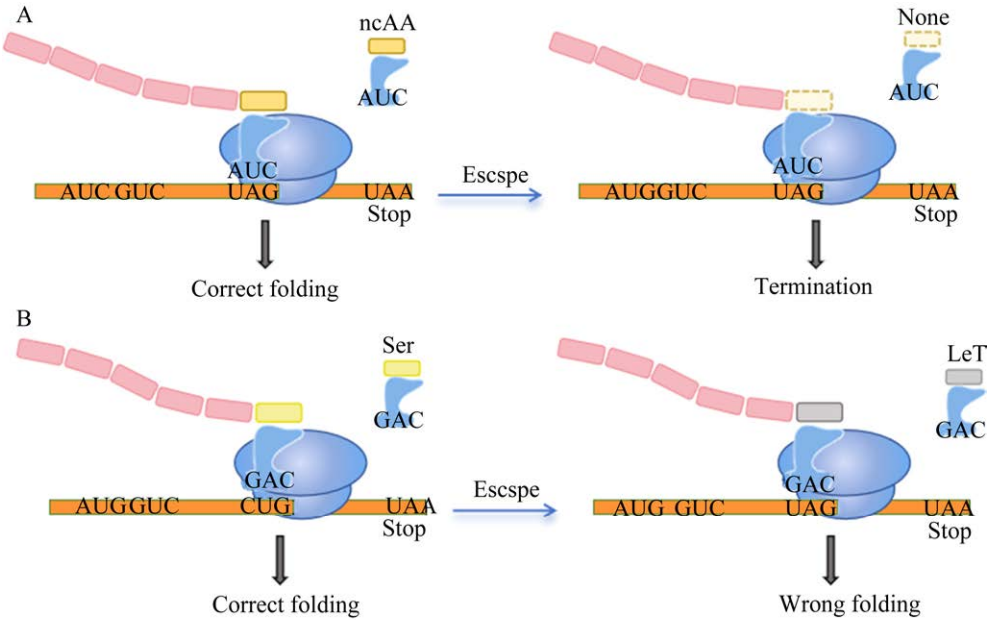

图 3　两种非天然氨基酸营养缺陷型菌株

Figure 3　Two non-natural amino acid auxotrophic strains. A: When non-canonical amino acids (ncAAs) are assigned to the stop codon UAG, upon the escape of the engineered organism (left), the absence of externally supplied ncAA will lead to the lethal premature termination of essential genes. B: The standard leucine CUG codon is decoded as serine (left), which leading to protein misfolding when the modified gene flows to other organisms.

酸，从而使蛋白功能恢复。用这样的方法改造必需基因，能使菌株的生长依赖于 ncAAs 的外部供应，达到生物遏制的效果。功能性四联体密码子作为遗传密码的扩展也提供了更丰富的替代方案[152]。研究人员还能利用合成基因组技术进行“密码子压缩”，在全基因组范围内用同义密码子替换目标密码子[153]。Venetz 等[154]在新月柄杆菌(*Caulobacter crescentus*) 785 701 bp 的基因组中引入了 133 313 个碱基替换，从而修改了 123 562 个密码子。

3.4 工程微藻生物封存技术展望

前沿生物技术在减少工程微藻的逃逸频率上有所成效，但仍会存在突破防线或系统失效的可能。如由敲除基因或者控制蛋白表达得到的营养缺陷型菌株，可能会通过获得其他生物体产生的基本代谢产物而继续生存，封存系统也可能因为基因水平转移回补得到被删除的基因而失效。采用毒素蛋白的自杀式防逃逸工程微藻，也可能由于毒素蛋白基因的突变而突破屏障。因此，需要不断地开发更多防止工程微藻逃逸的措施。

计算机技术的发展进一步推动了生物防护系统的模块化进程[155]，相关进展在生物封存技术领域的应用值得借鉴。Blazejewski 等[156]提出了一种通用策略，通过计算设计基因的重叠来保存和约束遗传信息。目标基因和必需基因的序列重叠能够改变其适应性，使工程基因的进化路径受阻。Blazejewski 等[156]利用这种方法将毒素基因嵌入目的序列中，缺乏抗毒素的受体会被杀死，而若是由于毒素基因突变而产生逃逸，其所携带的目标基因也会因为突变失去蛋白功能。这种方法目前还未在微藻之中开发，但其对于突变的鲁棒性有助于系统的长期运行，可能适用于工程微藻在燃料生产方面的应用。Chen 等[157]设计了模块化蛋白质，利用从头设计的蛋白质构建出各种逻辑门，实现复杂的翻译后逻辑控制。这种逻辑门可以作为致死开关的控制器，实现微藻的生物防护，同时模块化的设计也使之有潜力成为一种通用的遏制手段。Chavez 等[158]开发了一种突变的恢复系统，使用 Cas9 蛋白监测 DNA 序列中的特定变化，并将序列修改回初始组成，以防止负面突变的发生。这也是一种保持系统长期稳定的有效方法，并且具有一定的灵活性，适用于工程微藻的长期户外培养，形成自主维护。除此之外还可以建立多重防护体系，通过转录调控和 DNA 重组控制基本基因的表达这种双层控制来限制工程细胞，使之逃逸率降低到 10^{-10}/CFU 以下[159]。另外，在防逃逸菌种之外包裹水凝胶等物理防护措施[160]，也被认为是有效预防水平基因转移的新策略。

4 总结

目前，微藻凭借其丰富的能量储存和快速的生长速度，已成为绿色生物合成的重要底盘。前沿生物技术日益发展创新，进一步促进了微藻的研发与应用，但同时也带来了工程微藻扩散至自然界的风险。若想真正地将工程微藻从实验室菌株应用到工业生产中，必须考虑并解决这些问题。因此，利用日新月异的生物技术，建立有效的生物防护屏障十分必要。各种前沿生物技术的兴起和发展，为建立工程微藻的生物遏制体系带来了新的思路和更有效的手段，很大程度上推进了生物防护的发展。针对工程微藻建立生物遏制系统的开发还处于起步阶段，还需要研究人员集思广益、不断研发，继续从技术层面上减少工程微藻潜在的生物安全隐患。

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