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假单胞菌属脂肪酶的分子生物学研究进展*

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摘要 微生物脂肪酶是商品化脂肪酶的主要来源,并广泛应用于诸多工业领域。与其他微生物脂肪酶相比,细菌脂肪酶催化反应的类型更多、活性更高、稳定性更好,其中又以假单胞菌属(*Pseudomonas*)脂肪酶的性能最为优越。作为性能最为优越、应用最为广泛的一类脂肪酶,假单胞菌属脂肪酶研究一直是脂肪酶领域的热点。就假单胞菌属脂肪酶的分子生物学研究进展进行归纳和述评,包括基因资源挖掘及克隆、基因表达调控及分泌机制、活性过表达策略、蛋白质结晶及3D结构解析、蛋白质工程,并对其未来研究方向做出展望,以期为后续研究提供有益参考。

关键词 假单胞菌属 脂肪酶 基因表达调控

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脂肪酶(triacylglycerol acylhydrolase,EC 3.1.1.3)属于α/β型水解酶超家族,广泛存在于各种动物、植物和微生物中[1]。它们在油水界面上能催化长链酰基甘油水解为脂肪酸和二酰基甘油、一酰基甘油或甘油,而在微水相或非水相中能催化多种化学反应,如酯化反应、转酯反应、醇解反应、氨解反应、酸解反应等,催化的反应具有高的化学选择性、区域选择性和/或立体选择性[2-4]。由于微生物脂肪酶具有资源丰富、催化活性多样、遗传操作简单、产量较高、不随季节波动的稳定供应、生产周期短、生产成本低、稳定性好等优点,因此已成为商品化脂肪酶的主要来源,并被广泛应用于食品、饮料、油脂、洗涤剂、饲料、纺织、皮革、新型材料、精细化工、医药、化妆品、造纸、环境治理、生物能源等诸多工业领域[5-8]。而在各种来源的微生物脂肪酶中,以细菌脂肪酶催化反应的类型最多、活性最高、稳定性最好,其中又以假单胞菌属(*Pseudomonas*)脂肪酶的性能最为优越[4,9-10]。

假单胞菌属细菌是一类革兰氏阴性菌,属于γ-变形杆菌纲(Proteobacteria)。由于该属细菌广泛存在于自然界中,在微生物学发展早期就能观察到它们的存在,并一直成为科研人员的热门研究对象。该属细菌种类繁多、功能类群多样,有些是动物或植物病原体,如铜绿假单胞菌(*P. aeruginosa*)、丁香假单胞菌(*P. syringae*);有些对某些植物病害具有防治作用,如荧光假单胞菌(*P. fluorescens*)、防御假单胞菌(*P. protegens*)、绿针假单胞菌(*P. chlororaphis*)、橘黄假单胞菌(*P. aurantiaca*);有些具有生物降解能力,如产碱假单胞菌(*P. alcaligenes*)、门多萨假单胞菌(*P. mendocina*)、恶臭假单胞菌(*P. putida*);有些能引起食物腐败,如荧光假单胞菌、莓实假单胞菌(*P. fragi*)、隆德假单胞菌(*P. lundensis*)[11-13]。尽管假单胞菌属细菌分布广泛、功能类群多样,但大多能分泌脂肪酶以分解脂类物质供自身新陈代谢所需。作为性能最为优越、应用最为广泛的一类脂肪酶,对假单胞菌属脂肪酶在基因资源挖掘及克隆、基因表达调控及分泌机制、活性过表达策略、蛋白质结晶及3D结构解析、蛋白质工程等分子生物学研究方向上做了大量的研究工作,本文就这些研究方向进行归纳和述评,并对其未来研究方向进行展望。

1 基因资源挖掘及克隆

假单胞菌属脂肪酶基因的挖掘及克隆主要通过以下两种方法。

一种是基于基因组文库构建、活性筛选的方法,包括两种技术,即可培养微生物的基因组文库和不可培

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养微生物的宏基因组文库。采用基因组文库克隆微生物基因起始较早,已有多个研究小组克隆到假单胞菌属脂肪酶基因[11, 14-20]。宏基因组文库是一种能从不可培养微生物中克隆新基因的有效手段,据报道不可培养微生物超过原核生物总量的99%[21]。为从这一巨大资源中克隆新的假单胞菌属脂肪酶基因,Hardeman和Sjoling[21]、Khan和Jithesh[22]分别对海底沉积物和土壤开展了相关研究工作并取得了预期结果。

另一种是基于PCR的方法,包括两种技术,即基于基因组步移(genome walking)的二步法克隆基因全长和基于已报道假单胞菌属脂肪酶基因侧翼保守序列设计引物的一步法克隆基因全长。二步法首先也需要根据现有假单胞菌属脂肪酶基因的保守序列设计引物克隆到部分基因序列,然后根据这一序列采用基因组步移PCR即可获得基因全长[23-25]。随着数据库中假单胞菌属基因组序列和脂肪酶基因序列的日益增多,基于一步法克隆基因全长的研究越来越多[4, 10, 26-31],并表现出简单、快捷、成本低等优势。

2　基因表达调控及分泌机制

截止目前,已克隆了大量假单胞菌属脂肪酶基因,但对其表达调控机制的研究仍处于探索性阶段,主要集中在基于NtrB/C蛋白超家族的双组分系统(two-component system)[32-35]、基于酰基高丝氨酸内酯(acyl-homoserine lactones,acyl-HSL)的群体感应系统(quorum sensing system)[32, 36]、Gac/Rsm信号转导系统(Gac/Rsm signal transduction system)[36-39]、渗透压感应双组分系统EnvZ/OmpR[40]和精氨酸代谢调控因子ArgR[41]对脂肪酶基因的表达调控(图1)。从图1可知,只有Gac/Rsm信号转导系统的调控机制被阐明,通过途径①"Gac/Rsm信号转导系统→QS系统→NtrB/C蛋白超家族→脂肪酶"间接激活脂肪酶基因转录(发现于铜绿假单胞菌中[31-37],图2)或通过途径②"Gac/Rsm信号转导系统→脂肪酶"直接激活脂肪酶基因翻译(本课题组发现于防御假单胞菌中[39],图3)。然而,Gac/Rsm信号转导系统还通过其他未知途径对脂肪酶基因的表达进行微调,如抑制铜绿假单胞菌脂肪酶基因*lipA*的转录(图2)、激活防御假单胞菌脂肪酶基因*lipA*的转录(图3),虽然它们不占主导作用,但是调控的分子机制值得进一步研究。基于上述分析并结合相关文献[39, 42],我们对Gac/Rsm信号转导系统激活假单胞菌属脂肪酶基因表达的调控机制提出以下假设:当假单胞菌属细菌拥有基于acyl-HSL的QS系统时,主要通过途径①激活脂肪酶基因转录;当假单胞菌属细菌缺乏基于acyl-HSL的QS系统时,主要通过途径②激活脂肪酶基因翻译。

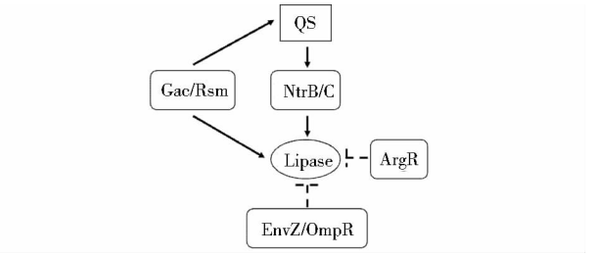

图1　调控假单胞菌属脂肪酶基因表达的调控因子

Fig. 1　Several regulators controlling expression of *Pseudomonas* lipase genes

Solid line: Direct regulation; Dotted line: Indirect regulation; Arrow: Positive effect; Bar: Negative effect

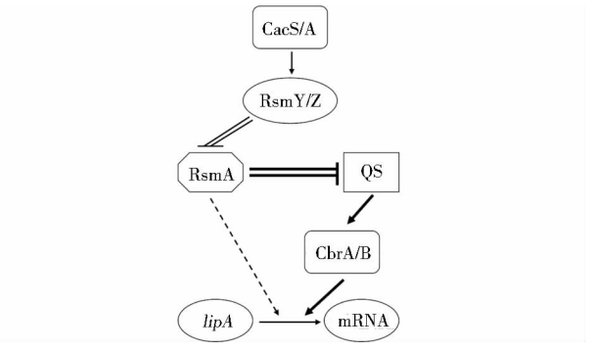

图2　铜绿假单胞菌中Gac/Rsm信号转导系统对*lipA*表达的调控

Fig. 2　Regulation of *lipA* expression by the Gac/Rsm signal transduction system in *P. aeruginosa*

Solid line: Direct regulation; Dotted line: Indirect regulation; Arrow: Positive effect; Bar: Negative effect; Double line: Physical interaction

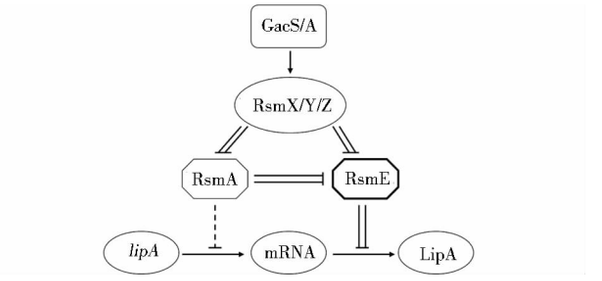

图3　防御假单胞菌中Gac/Rsm信号转导系统对*lipA*表达的调控

Fig. 3　Regulation of *lipA* expression by the Gac/Rsm signal transduction system in *P. protegens*

Solid line, direct regulation; dotted line, indirect regulation; arrow, positive effect; bar, negative effect; double line, physical interaction

假单胞菌属细菌是一类革兰氏阴性菌，主要通过两种途径分泌胞外脂肪酶（表 1）[6, 32]。Type I 型分泌途径：脂肪酶 C 端信号序列首先被 ABC 输出子特异性识别，然后通过 ABC 蛋白复合体形成的通道直接分泌到胞外。Type II 型分泌途径：脂肪酶前体 N 端信号肽首先被内膜（质膜）上的多亚基蛋白复合体 Sec 装置识别并进入周质，此过程伴随信号肽的切除；然后在同源折叠酶 Lif 和二硫键形成蛋白 Dsb 的作用下折叠成正确构象并形成二硫键，此过程中错误折叠的脂肪酶将被周质蛋白酶所降解；成熟脂肪酶最后在 Xcp 分泌子的介导下通过外膜分泌到胞外。

表 1 假单胞菌属脂肪酶的分泌途径

Table 1 Secretion pathways of *Pseudomonas* lipases

Secretion pathways	Secretion systems	Secretion signals	Accessory factors	Representative lipases
Type I	ABC 输出子：由内膜 ATP 酶、膜融合蛋白 MFP 和外膜蛋白 OMP 组成	C 端信号序列，不被切除	无	荧光假单胞菌 SIK W1 脂肪酶
Type II	内膜 Sec 装置：由多个亚基组成；内外膜 Xcp 分泌子：由≤ 14 个蛋白质组成	N 端信号肽，被切除	Lif、Dsb	铜绿假单胞菌 PAO1 脂肪酶

3 活性过表达策略

脂肪酶的大量活性表达一直是困扰科研人员的难题，其原因主要有两：其一，宿主细胞常常没有与异源脂肪酶相对应的分泌系统，大量表达的异源脂肪酶容易对宿主细胞产生较大毒性而过早死亡，或者为避免毒害宿主细胞而形成无活性包涵体；其二，多数脂肪酶需要在同源折叠酶的作用下才能形成正确构象的活性蛋白质，而异源宿主细胞常常没有对应的折叠酶[43]。由于假单胞菌属脂肪酶的优越性，人们对其活性过表达开展了一系列研究，归纳起来主要有以下几种策略。

3.1 脂肪酶基因与同源分泌系统基因簇共表达

Ahn 等[44]将荧光假单胞菌 SIK W1 的脂肪酶基因 *tliA* 及同源 ABC 输出子基因簇 *tliDEF* 在假单胞菌属菌株中共表达，研究了同源分泌系统对假单胞菌属细菌产脂肪酶的影响。结果表明，共表达 *tliDEFA* 显著性优于单独表达 *tliA*，其中同源表达 *tliDEFA* 效果最好，是同源表达 *tliA* 的 69 倍；同源共表达显著性优于异源共表达，但同源表达 *tliA* 不一定优于异源表达 *tliA*；TliDEF 介导的 TliA 分泌具有温度依赖性，TliDEF 在培养温度高于 30℃时丧失分泌功能。然而，并不是所有同源共表达都优于异源共表达。Song 等[45]的研究表明，*tliDEFA* 在黏质沙雷氏菌（*Serratia marcescens*）中共表达显著性优于同源共表达，可能是因为 TliDEF 介导的 TliA 分泌在黏质沙雷氏菌中没有生长阶段依赖性，能持续表达并分泌 TliA 直至稳定生长期。对于 Type II 型分泌途径分泌的脂肪酶，*xcp* 基因簇的过表达能增加脂肪酶的产量[46-47]。

3.2 脂肪酶基因与同源折叠酶基因共表达

多数假单胞菌属脂肪酶表达后，需要在同源折叠酶的作用下才能形成活性蛋白质，它们的异源表达经常形成无活性包涵体。大量研究表明，假单胞菌属脂肪酶基因与同源折叠酶基因的共表达能很好地克服这一瓶颈[15, 29, 48-50]。Omori 等[48]以假单胞菌属菌株 109 的脂肪酶操纵子 *lipL/limL* 为研究对象，系统研究了表达宿主、启动子、基因拷贝数及其组合对 *lipL/limL* 共表达的影响。结果显示：在弱启动子 *tac-lac* UV5 的控制下，同源表达产脂肪酶的效果是原始菌株的 7.1 倍；在强启动子 T7 的控制下，铜绿假单胞菌中异源表达产脂肪酶的效果是原始菌株的 13 倍；*lipL* 和/或 *limL* 基因拷贝数的组合也会影响脂肪酶产量，其中 $(lipL)_3$-*limL* 组合最优，其产脂肪酶的效果是原始菌株的 67 倍。因此，表达宿主适配性、启动子强弱、共表达基因的拷贝数及组合都是影响脂肪酶产量的主要因素。

3.3 脂肪酶表面展示

Lee 等[51-52]利用铜绿假单胞菌外膜蛋白 OprF 成功将 TliA 展示在大肠杆菌（*Escherichia coli*）XL10-Gold 和恶臭假单胞菌 KT2442 表面，与大肠杆菌表面展示系统相比，恶臭假单胞菌表面展示系统在酶活力、有机溶剂耐受性、温度稳定性、手性拆分能力等方面表现出更大优势。Jung 等[53-54]利用丁香假单胞菌冰核蛋白 INP 将 TliA 成功展示在大肠杆菌 JM109 和恶臭假单胞菌 GM730 表面，恶臭假单胞菌表面展示系统也全面优于大肠杆菌表面展示系统。

3.4 脂肪酶基因在酵母中表达

与大肠杆菌、酿酒酵母（*Saccharomyces cerevisiae*）表

达系统相比,巴斯德毕赤酵母(*Pichia pastoris*)表达系统具有无可比拟的优势[55],作为异源宿主也常常被应用于表达原核基因。Jiang 等[23]、王海燕[55]、Yang 等[27]利用巴斯德毕赤酵母作为异源宿主成功实现了荧光假单胞菌脂肪酶基因的表达和分泌,这是因为该类脂肪酶属于 I.3 亚家族,无需同源折叠酶等辅助因子的帮助就能折叠成活性构象。

4　蛋白质结晶及 3D 结构解析

脂肪酶 3D 结构在理论方面有助于解释催化机制和研究构效关系,在工程技术方面可以辅助蛋白质工程。到目前为止,有三个假单胞菌属脂肪酶的 3D 结构被解析。

Boston 等[56]解析了门多萨假单胞菌脂肪酶的 3D 结构(PDB ID:2FX5),并基于结构分析研究了点突变对脂肪酶活力的影响,认为增加甘油三酯底物的酰基链结合是提高脂肪酶活力的关键。Sibille 等[57]在此基础上,通过 NMR 分析和分子动力学模拟提出了降低脂肪酶的分子柔性是提高其稳定性的基础。

Nardini 等[58]解析了铜绿假单胞菌 PAO1 脂肪酶(PAL)开放构象的 3D 结构(PDB ID:1EX9),根据结构分析得出酰基口袋大小、*sn*-2 结合口袋大小及相互作用是 PAL 具有区域和立体选择性的决定性因素。

Angkawidjaja 等[59]解析了假单胞菌属菌株 MIS38 脂肪酶(PML)及其铂结合 S445C 突变体封闭构象的 3D 结构(PDB ID:2Z8X、2Z8Z),发现 PML 有两个盖子结构(Lid1 和 Lid2)。基于结构分析得出了盖子结构的开放机制,即 Lid1 在开放构象时,Ca^{2+}结合到 Ca1 有助于 Lid1 定位于开放构象;Lid1 在封闭构象时,Ca1 消失、Lid1 的结构和位置发生巨大改变;Lid2 在开放构象时形成一个螺旋状发夹结构,而在封闭构象时不形成该发夹结构并覆盖活性位点。为研究 Ca^{2+}结合位点对 PML 活力和稳定性的重要性,该研究小组解析了两个突变体 D157A 和 D337A 的 3D 结构(PDB ID:2ZJ6、2ZJ7),结果表明活性位点的完全开放需要 Ca1、Ca2 位点,Ca2、Ca3 位点有助于 PML 的稳定[60]。该研究小组又根据 PML 不同构象的 3D 结构(PDB ID:2ZVD、3A6Z、3A70)结合分子动力学模拟研究了 PML 的界面激活机制,结果表明 Ca1 的功能类似于钩子,有助于稳定 Lid1 的完全开放构象和引发 Lid2 的随后开放[61]。

5　蛋白质工程

蛋白质工程以基因工程为基础,根据蛋白质的结构和功能,通过基因的人工定向改造获得性质改变的蛋白质以满足人类需求。蛋白质工程主要有三种技术,分别为理性设计(rational design)、非理性设计(irrational design)和半理性设计(semi-rational design)[62]。

5.1　理性设计

理性设计需要蛋白质的 3D 结构和功能信息,通过分析结构与功能的关系确定需要突变的氨基酸残基,最后利用定点突变技术达到突变氨基酸残基的目的[63]。Santarossa 等[64]通过理性设计定点突变了莓实假单胞菌脂肪酶(PFL)盖子区的 3 个极性氨基酸残基,T137 和 T138 的突变增加了 PFL 对 C8 底物的相对活力和 PFL 的温度稳定性,而 S141 的突变破坏 PFL 的稳定。

5.2　非理性设计

非理性设计又称为定向进化或体外分子进化,不需要蛋白质的 3D 结构和功能信息,通过模拟自然进化随机突变目的基因,定向筛选后获得预期的改良蛋白质[65]。Jung 等[53]利用非理性设计随机突变 *tliA*,通过三丁酸甘油酯平板筛选得到 4 个水解活力增高的 TliA 突变体(T48、T54、T61 和 T68)。Fujii 等[66]随机突变铜绿假单胞菌脂肪酶基因,通过油酰基-2-萘酰胺(oleoyl 2-naphthylamide)水解活力筛选得到 5 个酰胺水解酶活力增高的突变体,其中 3 个突变位点(F207S、A213D 和 F265L)影响酰胺水解酶/酯酶活力比;这些突变位点的组合进一步提高酰胺水解酶活力,其中 F207S/A213D 组合的活力最高;3D 结构模拟显示这些突变位点远离活性中心而靠近蛋白质表面的 Ca^{2+}结合位点。

5.3　半理性设计

半理性设计是理性设计与非理性设计的结合,融合了理性设计和非理性设计的优点,需要蛋白质的 3D 结构或功能信息。一般来说,半理性设计包括 4 种方案:对特定氨基酸残基进行随机突变、随机突变与定点饱和突变同时进行、随机突变筛选突变位点后进行定点饱和突变、利用计算方法进行半理性设计和组合设计[62]。Carballeira 等[67]采用组合活性中心饱和试验(combinatorial active site saturation test,CAST)的策略定向筛选到一个具有高度对应选择性的铜绿假单胞菌脂肪酶突变体(Leu162Phe)。Nakagawa 等[68]利用随机突变和饱和突变相结合的策略定点突变 *P. aeruginosa* 脂肪酶底物结合位点的所有氨基酸残基,鉴定出残基 Leu252 是提高酰胺水解酶活力的热点,Met 或 Phe 替换

Leu252 显著性增加该脂肪酶的酰胺水解酶活力而酯酶活力不受影响，其中三联突变体（F207S/A213D/M252F）的酰胺水解酶活力是原始酶的 28 倍。

可以预期，随着脂肪酶 3D 结构解析的日益增多，理性设计和半理性设计将会成为脂肪酶蛋白质工程的首选技术。

6 展 望

假单胞菌属脂肪酶性能优越、应用范围广泛，在生物转化领域具有非常重要的作用，一直是脂肪酶研究领域的热点对象。虽然已在前文所述分子生物学研究方向上开展了大量研究，并取得了丰硕成果，但是在工业化生产、基因资源挖掘及克隆、蛋白质结晶及 3D 结构解析、生产系统构建等方向仍需大量而深入的研究，具体研究内容见表 2。

表 2 假单胞菌属脂肪酶的未来研究方向

Table 2 Future research directions of *Pseudomonas* lipases

Research directions	Research contents
工业化生产	基因表达调控机制、基因工程菌构建、发酵条件优化
基因资源挖掘及克隆	极端环境微生物脂肪酶基因、宏基因组筛选不可培养微生物脂肪酶基因、基因组数据库中的脂肪酶基因、蛋白质工程改造现有脂肪酶基因
蛋白质结晶及 3D 结构解析	解释催化机制、研究构效关系、辅助蛋白质工程
生产系统构建	用于不同来源脂肪酶的表达与分泌

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Research Advances in Molecular Biology of *Pseudomonas* Lipases

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Abstract　Microbial lipases, being major sources of commercial ones, which have been widely utilized in many industrial fields, such as foods, beverages, lipids, detergents, feeds, textiles, leathers, advanced materials, fine chemicals, medicines, cosmetics, papermaking, pollution treatment, and bioenergy, etc. Compared with other microbial lipases, bacterial lipases have more types of reactions and exhibit higher activity and better stability in many reactions. Amongst bacterial lipases, the most excellent ones are those being from the genus *Pseudomonas*. As one of the most excellent and the most widely used lipases, the study on *Pseudomonas* lipases has been a hot topic in the field of lipases. Research advances in molecular biology of *Pseudomonas* lipases, including mining and cloning of genetic resources, molecular mechanisms of gene expression regulation and protein secretion, strategies for efficient overexpression, protein crystallization and 3D structure analysis, and protein engineering, were summarized and reviewed. Furthermore, the future research directions of *Pseudomonas* lipases were prospected so as to provide a useful reference for the follow-up studies.

Key words　*Pseudomonas*　Lipase　Gene expression and regulation