嗜水气单胞菌合成含 3-羟基戊酸单体的聚羟基脂肪酸共聚酯的研究

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摘 要 分别利用葡萄糖或葡萄糖酸钠与十一碳酸、月桂酸与十一碳酸为混合碳源进行嗜水气单胞菌(*Aeromonas hydrophila*)菌株 4AK4 的摇瓶培养,实现了含有 3-羟基戊酸(3HV)单体的聚羟基脂肪酸酯的微生物合成。当使用葡萄糖或葡萄糖酸钠与十一碳酸为混合碳源时,野生型 *A. hydrophila* 4AK4 及含有 3-羟基丁酸辅酶 A 合成基因 *phaA* 和 *phaB* 的重组 *A. hydrophila* 4AK4 (pTG01)能够合成 3-羟基丁酸(3HB)与 3HV 的共聚物,且葡萄糖或葡萄糖酸钠与十一碳酸比例为 1:1 时最利于细胞生长和 PHA 的积累。当使用月桂酸和十一碳酸为混合碳源时,*A. hydrophila* 4AK4 能够合成 3HB、3HV 与 β-羟基己酸(3HHx)的共聚物,且随着混合碳源中十一碳酸的含量增加,*A. hydrophila* 4AK4 合成的 PHA 中 3HV 的比例增加,而 3HB 和 3HHx 的比例降低。

关键词 聚羟基脂肪酸酯, PHA, 嗜水气单胞菌

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聚羟基脂肪酸酯(Polyhydroxyalkanoate 简称为 PHA)是一种可由多种微生物合成的生物聚酯[1-3]。1926 年 Lemoigne 首先在 *Bacillus megaterium* 中发现聚 β-羟基丁酸酯(简称 PHB)[4],从那时起,各种不同的 PHA 被陆续报道。PHB 和 3-羟基丁酸与 3-羟基戊酸的共聚物(PHBV)是 PHA 中研究最多的两种聚合物,*Ralstonia eutropha*(原来被称为 *Alcaligenes eutrophus*), *Alcaligenes latus* 和 *Azotobacter vinelandii* 都能够利用糖类和丙酸或戊酸进行 PHB 及 PHBV 的合成[5-7]。

嗜水气单胞菌(*Aeromonas hydrophila*)能合成 3-羟基丁酸与 3-羟基己酸的共聚物(PHBHHx)。由于其含有中等碳链长度的 3-羟基己酸(3HHx)单体,因而材料脆性降低,柔韧性增加,具有更好的可加工性[8],目前已成为研究的热点[9-12]。*A. hydrophila* 4AK4 是其中有较强 PHBHHx 合成能力的一株。*A. hydrophila* 中合成 PHBHHx 所需要的前体仅依赖于脂肪酸的 β-氧化过程提供[9,12],不能利用非相关性碳源(如糖类)合成 PHBHHx。但到目前为止还没有关于 *A. hydrophila* 利用奇数碳原子数的脂肪酸为底物合成 PHA 的报道。本研究以多种不同碳链长度的奇数碳原子数的脂肪酸和糖类或月桂酸为底物共同培养 *A. hydrophila* 4AK4,研究了其合成含有奇数碳原子数单体的 PHA 的能力。

本研究还在 *A. hydrophila* 4AK4 中引入了 β-酮基硫解酶基因(*phaA*)和乙酰乙酰辅酶 A 还原酶基因,β-酮基硫解酶能够催化两个乙酰辅酶 A 缩合生成乙酰乙酰辅酶 A;乙酰乙酰辅酶 A 还原酶能够将乙酰乙酰辅酶 A 转化为 3-羟基丁酰辅酶 A(3HB-CoA)[1,13]。这条新的提供 3HB-CoA 的代谢途径使重组 *A. hydrophila* 4AK4 可以利用 β-氧化途径或糖类代谢途径产生的乙酰辅酶 A 合成 3-羟基丁酸(3HB)前体,并能由此提高所合成的聚合物中 HB 单体的组成比例。

1 材料和方法

1.1 菌株

Aeromonas hydrophila 4AK4 为清华大学微生物实验室收藏。

1.2 重组菌株的构建

带有 *phbA* 和 *phbB* 基因的质粒 pTG01 构建方法参见参考文献[14],通过接合转化的方法将质粒

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pTG01 转入到 *A. hydrophila* 4AK4 中。

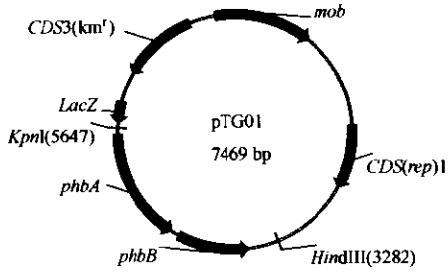

图 1 pTG01 质粒图

Fig.1 Plasmid map of pTG01

1.3 摇瓶培养基及培养条件

A. hydrophila 4AK4 的培养基组成(g/L): $(NH_4)_2SO_4$ 2, $MgSO_4 \cdot 7H_2O$ 0.41, $Na_2HPO_4 \cdot 12H_2O$ 9.0, KH_2PO_4 1.5, Fe(Ⅲ)-NH_4-Citrate 0.05, $CaCl_2 \cdot 2H_2O$ 0.02 和微量元素溶液 1 mL/L。微量元素溶液含有(g/L): $CuSO_4 \cdot 5H_2O$ 0.01, $MnCl_2 \cdot 4H_2O$ 0.03, $ZnSO_4 \cdot 7H_2O$ 0.1, H_3BO_3 0.3, $CoCl_2 \cdot 6H_2O$ 0.2, $NiCl_2 \cdot 6H_2O$ 0.02 和 $NaMnO_4 \cdot 2H_2O$ 0.03。卡那霉素50μg/mL用于保持质粒的稳定。各种碳源的用量见结果。

A. hydrophila 4AK4 的培养条件为:30℃,24h,摇床转速为 200r/min (NBS Series 25D, New Brunswick, NJ, 美国)。

1.4 分析方法

细胞干重(Cell Dry Weight)CDW、PHA 含量测量方法见文献[15],数据由 3 个平行样品获得。

2 结 果

2.1 *A. hydrophila* 4AK4 在不同奇数碳原子数脂肪酸上的生长及 PHA 的积累

野生型 *A. hydrophila* 4AK4 利用糖类等非相关性碳源只能积累痕量的 PHB,当利用葡萄糖酸钠和不同碳原子数的奇数碳脂肪酸作为共底物进行培养,结果表明细菌不能在碳原子数小于 11 的奇数碳脂肪酸上生长,而能够在葡萄糖酸钠和十一碳酸的混合碳源中生长并利用十一碳酸为底物合成 PHA,其单体组成以 3-羟基戊酸(3HV)为主(表 1),含有极少量的 3HB 单体。

表 1 *A. hydrophila* 4AK4 在葡萄糖酸钠和不同奇数碳原子数脂肪酸上的生长及 PHBV 的积累

Table 1 PHBV Production by *A. hydrophila* 4AK4 in different fatty acids with odd carbon number

Carbon source	Conc./(g/L)	CDW/(g/L)	(PHA/CDW)/(wt%)	(3HV/PHA)/(wt%)
Gn + PA	2 + 2	NG	-	-
Gn + VA	2 + 2	NG	-	-
Gn + HA	2 + 2	NG	-	-
Gn + NA	2 + 2	NG	-	-
Gn + UA	2 + 2	1.14 ± 0.14	61.41 ± 3.12	98.62 ± 0.11

"NG" Not growth; " - " Not detected;

PA: Propionic acid; VA: Valeric acid; HA: Heptanoic acid; NA: Nonanoic acid; UA: Undecanoic acid; Gn: Gluconate

2.2 *A. hydrophila* 4AK4 及重组 *A. hydrophila* 4AK4 (pTG01)在糖类和十一碳酸混合碳源上的生长及 PHA 的积累

本研究还在 *A. hydrophila* 4AK4 中引入了 β-酮基硫解酶基因(*phaA*)和乙酰乙酰辅酶 A 还原酶基因(*phaB*),构建了重组 *A. hydrophila* 4AK4(pTG01)。以十一碳酸和葡萄糖或葡萄糖酸钠为混合碳源培养野生菌和重组菌,结果显示,葡萄糖酸钠总体上说来更适于野生菌的培养和 PHA 的积累,CDW 及 PHA 的含量都相对较高。而对重组菌而言,葡萄糖更利于细菌的生长,但对 PHA 的积累没有明显优势(表 2)。

两种碳源的比例是影响 PHA 积累量的重要因素,由表 2 中的结果可知,用 1:1 的葡萄糖或葡萄糖酸钠与十一碳酸培养野生菌时最利于 PHA 的积累,当两者比例为 1:3 或者 3:1 时,细胞积累的 PHA 明显减少。碳源的比例对共聚物中单体的组成比例也有一定影响,实验发现当葡萄糖或葡萄糖酸钠与十一碳酸的比例为 1:1 时,合成的 PHBV 中 3HB 单体的含量最低,质量百分含量小于 2%,而当两者比例为 1:3 或者 3:1 时,合成的 PHBV 中 3HB 单体的含量均在 4% 以上。但总体而言,在葡萄糖或葡萄糖酸钠与十一碳酸为混合碳源时合成的 PHBV 中均以 3HV 单体为主。

对重组菌而言,当利用 1:1 的葡萄糖或葡萄糖酸钠与十一碳酸为碳源时,其合成的 PHBV 中 HB 单体的比例有明显提高。由下表的数据可知在 *A. hydrophila* 4AK4 中葡萄糖或葡萄糖酸钠和十一碳酸

为混合碳源共同培养时细菌合成PHA的能力没有明显差别,但单体的组成比例略有差异。当以葡萄糖酸钠为底物时,合成的PHBV中3HB单体的质量百分含量为34.41%,而葡萄糖为底物时3HB单体的质量百分含量为29.26%。

2.3 *A. hydrophila* 4AK4在十一碳酸及月桂酸上的生长及PHA的积累

利用月桂酸和十一碳酸作为混合碳源培养*A. hydrophila* 4AK4,得到了3HB、3HV和3HHx三种单体的共聚物(结果见表3及图3)。由结果可知在培养24h后,随着混合碳源中十一碳酸的含量增加,*A. hydrophila* 4AK4的生长情况渐差,细胞干重逐渐降低。但PHA的含量变化受碳源比例变化的影响不大。

图3中的结果表明随着混合碳源中十一碳酸的含量增加,*A. hydrophila* 4AK4合成的PHA中3HV的比例增加,而3HB和3HHx的比例降低,培养时间对各种单体的组成比例无明显影响。

表2　*A. hydrophila* 4AK4和*A. hydrophila* 4AK4(pTG01)在糖类和十一碳酸混合碳源上的生长及PHA的积累

Table 2　PHBV Production by *A. hydrophila* 4AK4 and *A. hydrophila* 4AK4 (pTG01) in undecanoic acid and glucose/gluconate

Strains	Carbon source	Conc./(g/L)	CDW/(g/L)	(PHA/CDW)/(wt%)	(3HV/PHA)/(wt%)
A. hydrophila 4AK4		1+3	0.68±0.06	6.49±1.00	94.5±3.44
	Glu + UA	2+2	0.80±0.21	42.2±6.43	98.3±0.06
		3+1	1.35±0.10	2.91±0.95	94.8±1.12
		1+3	0.53±0.02	11.4±0.18	95.3±0.21
	Gn + UA	2+2	1.14±0.14	61.4±3.12	98.6±0.11
		3+1	1.48±0.03	1.37±0.16	92.4±1.74
A. hydrophila 4AK4 (pTG01)	Glu + UA	2+2	0.90±0.06	34.8±1.90	70.7±1.40
	Gn + UA	2+2	0.65±0.04	35.2±2.49	65.6±2.85

UA: Undecanoic acid; Gn: Gluconate; Glu: Glucose

表3　*A. hydrophila* 4AK4在十一碳酸和月桂酸混合碳源上的生长及PHA的积累

Table 3　PHBV Production by *A. hydrophila* 4AK4 in undecanoic acid and lauric acid

Carbon source	Conc./(g/L)	t/h	CDW/(g/L)	(PHA/CDW)/(wt%)
LA + UA	3+1	24	1.58±0.30	47.26±2.65
	2+2	24	1.02±0.02	49.52±0.79
	1+3	24	0.36±0.04	43.32±2.07

UA: Undecanoic acid; LA: Lauric acid

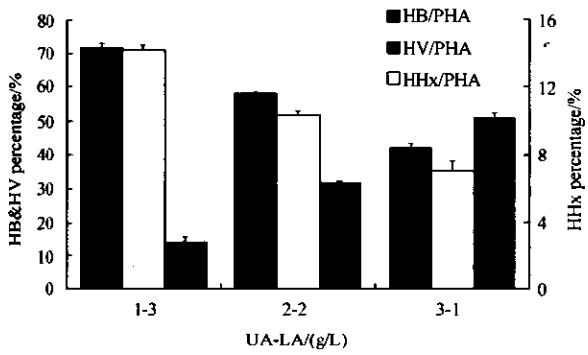

图2　*A. hydrophila* 4AK4在月桂酸和十一碳酸混合碳源上合成的PHA中单体组成的比例

Fig.2　PHA monomer composition produced by *A. hydrophila* 4AK4 when grown in undecanoic acid and lauric acid

3　讨　论

野生型*A. hydrophila* 4AK4能够在月桂酸上很好地生长并积累PHBHHx[10],由于其PHA合成酶的底物专一性范围较宽,能够将3HB和3HHx单体聚合形成共聚物。*A. hydrophila* 4AK4主要依赖β-氧化过程产生PHA合成的前体,3HB和3HHx单体的前体均来源于月桂酸(十二碳酸)β-氧化的中间产物。由此推测如果使用奇数碳原子数的脂肪酸,则可能能够有奇数碳原子数的单体前体产生并被聚合至PHA中。野生型*A. hydrophila* 4AK4在糖类等非相关性碳源上生长时只能积累痕量的PHB。当利用葡萄糖酸钠和不同碳原子数的奇数碳脂肪酸作为共底物培养*A. hydrophila* 4AK4时,结果显示*A. hydrophila* 4AK4不能在碳原子数小于11的奇数碳脂

肪酸上生长,这是由于短链的脂肪酸对细菌有毒害作用的缘故。细菌以十一碳酸为底物时生长极差(数据未显示),而在葡萄糖酸钠和十一碳酸的混合碳源中生长良好并能够利用十一碳酸为底物合成PHA,其单体组成以3HV为主(表1),含有极少量的3HB单体。这一结果也表明 *A. hydrophila* 4AK4 中合成 PHA 的前体主要来源于脂肪酸 β-氧化途径,通过糖类代谢提供 PHA 合成前体的能力极弱。

A. hydrophila 4AK4 在葡萄糖或葡萄糖酸钠上生长良好,而十一碳酸是一种能够为 *A. hydrophila* 4AK4 提供 PHA 合成前体的碳源。这两种碳源作为共底物培养 *A. hydrophila* 4AK4 时,能够相辅相成,通过糖类碳源提供能量支持细菌的生长代谢,而十一碳酸则主要作为提供 PHA 合成前体的物质。本文中以十一碳酸为脂肪酸碳源,探讨了葡萄糖和葡萄糖酸钠两种糖类碳源对 PHA 合成的不同影响以及两种碳源的不同比例对合成 PHA 的合成的影响。从结果可以看出,两种碳源的比例是影响 PHA 积累量的重要因素,当葡萄糖或葡萄糖酸钠与十一碳酸的比例为 1:1 时最利于 PHA 的积累,当两者比例为 1:3 或者 3:1 时,细胞积累的 PHA 明显减少。由于糖类的代谢对脂肪酸的 β-氧化途径有一定的抑制作用[16],当糖类碳源浓度大于脂肪酸碳源浓度时,细菌主要利用糖类碳源提供能量生长,较少利用脂肪酸碳源,脂肪酸 β-氧化途径不活跃,因而通过 β-氧化途径提供的 PHA 合成前体较少,PHA 的积累量也较低。而当糖类碳源浓度较低而十一碳酸浓度高时,细菌不能利用十一碳酸很好的生长,因而细胞干重和 PHA 的积累量都较低。此外,碳源的比例对共聚物中单体的组成比例也有一定影响,当葡萄糖或葡萄糖酸钠与十一碳酸的比例为 1:1 时,合成的 PHBV 中 3HB 单体的含量最低,而当两者比例为 1:3 或者 3:1 时,合成的 PHBV 中 3HB 单体的含量均在 4% 以上。碳源的比例不同会影响糖代谢途径和脂肪酸 β-氧化途径的相对活性,则通过糖代谢途径和脂肪酸 β-氧化途径提供 PHA 合成前体的能力在不同的碳源浓度下有所差异,因而合成的共聚物中单体比例不同。

本研究还在 *A. hydrophila* 4AK4 中引入了 β-酮基硫解酶基因(phaA)和乙酰乙酰辅酶 A 还原酶基因(phaB)。PhaA 能够催化两个乙酰辅酶 A 缩合生成乙酰乙酰辅酶 A;PhaB 能够将乙酰乙酰辅酶 A 转化为 3-羟基丁酰辅酶 A(3HB-CoA)[1]。这条新的提供 3HB-CoA 的代谢途径使重组 *A. hydrophila* 4AK4 可以利用 β-氧化途径或糖类代谢途径产生的乙酰辅酶 A 合成 3HB 前体,用 1:1 的葡萄糖或葡萄糖酸钠与十一碳酸为碳源培养重组 *A. hydrophila* 4AK4 (pTG01)时,其合成的 PHBV 中 HB 单体的比例明显高于野生型 *A. hydrophila* 4AK4。且葡萄糖或葡萄糖酸钠和十一碳酸为混合碳源共同培养重组 *A. hydrophila* 4AK4(pTG01)时,细菌合成 PHA 的能力没有明显差别,但单体的组成比例略有差异。以葡萄糖酸钠为底物时,合成的 PHBV 中 3HB 单体的质量百分含量高于以葡萄糖为底物时 3HB 单体的质量百分含量,这可能是由于葡萄糖和葡萄糖酸钠在 *A. hydrophila* 4AK4 中的代谢差异造成的,由于目前还没有关于 *A. hydrophila* 基础代谢背景方面的系统研究的报道,其代谢差异的具体机制仍需进一步的研究。

本研究中利用月桂酸和十一碳酸作为混合碳源培养 *A. hydrophila* 4AK4,得到了 3HB、3HV 和 3HHx 三种单体的共聚物,且随着混合碳源中十一碳酸的含量增加,*A. hydrophila* 4AK4 合成的 PHA 中 3HV 的比例增加,而 3HB 和 3HHx 的比例降低,说明其单体的含量与所使用相应碳源的比例相关,通过调节混合碳源的比例关系,可以控制产物中各种单体的组成比例。

本文分别利用葡萄糖或葡萄糖酸钠与十一碳酸、月桂酸与十一碳酸为混合碳源进行培养第一次在嗜水气单孢菌中实现了含有 3HV 单体的聚羟基脂肪酸酯的微生物合成,且共聚物中单体的比例可以通过调节培养基中各种碳源的浓度而调控。

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Studies on Synthesis of Polyhydroxyalkanoate Consisting of 3-hydroxyvalerate by *Aeromonas hydrophila*

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Abstract Polyhydroxyalkanoates (PHA) is a family of microbially synthesized polyesters consisting of various 3-hydroxyalkanoate monomers. *Aeromonas hydrophila* 4AK4 could be able to synthesize PHA copolymer consisting of 3-hydroxybutyrate (3-HB) and 3-hydroxyhexanoate (3-HHx). No data has been reported about the ability to synthesize the PHA with other monomers in *A. hydrophila*. In this study, propionic acid, valeric acid, heptanoic acid, nonanoic acid and undecanoic acid were used together with gluconate to find out whether *A. hydrophila* 4AK4 could synthesize the PHA consisting of odd carbon atom number monomers. The result showed that *A. hydrophila* 4AK4 could not growth when supplied with propionic acid, valeric acid, heptanoic acid and nonanoic acid and only undecanoic acid could be used to synthesize PHA. Wild type and recombinant *A. hydrophila* 4AK4 harboring *phaA*(β-ketothiolase) and *phaB*(acetoacetyl-CoA reductase) were cultivated with undecanoic acid and glucose or undecanoic acid and gluconate served as carbon sources. PHA consisting of 3-HB and 3-hydroxyvalerate (3-HV) could be produced by both wild type and recombinant *A. hydrophila* 4AK4 and the latter could produce PHA with more 3-HB monomer. When the ratio of glucose or gluconate to undecanoic acid was 1:1, the cell dry weight (CDW) of *A. hydrophila* 4AK4 reached 1.14 g/L and PHA content was 60% of the CDW after cultivation for 24 h. When lauric acid and undecanoic acid were served as co-substrate, *A. hydrophila* 4AK4 could produce copolyester consisting of 3-HB, 3-HV and 3-HHx. Along with the increase of undecanoic acid proportion in the mixed carbon source, the 3-HV content of copolymer was increased while the 3-HB and 3-HHx content were decreased. In all cases, the CDW decreased along with the increase of undecanoic acid concentration, which indicated that undecanoic acid was not very good for *A. hydrophila* 4AK4 growth.

Key words polyhydroxyalkanoates, PHA, *Aeromonas hydrophila*

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Influence of Phospholipid Fatty Acid Composition of Plasma Membrane on Sensitivity of Plasma Membrane ATPase of a Self-flocculating Yeast to *in vivo* Ethanol Activation and its Relationship to Ethanol Tolerance

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Abstract Although alterations in fatty acid composition of phospholipids in plasma membranes had no effect on activities of plasma membrane ATPases of a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* cells grown in the absence of ethanol (basal enzymes), they significantly affected the susceptibilities of the enzymes to *in vivo* activation induced by ethanol: the maximal values for the activated enzymes in cells pregrown with 0.6 mmol/L palmitic, linoleic or linolenic acid respectively were 3.6, 1.5 and 1.2-fold higher than their respective basal levels (in cells grown without ethanol), whereas the corresponding value for cells pregrown in the absence of fatty acid was 2.3-fold, with the concentrations of ethanol for the above maximal *in vivo* activation of enzymes being 7%, 6%, 6% and 7% (*V*/*V*) respectively. The K_m values for ATP, the pH profiles, and the sensitivities to orthovanadate of the basal and the activated plasma membrane ATPases were essentially identical; however, the v_{max} values of activated enzymes increased significantly. It was found that the characteristics of phospholipid fatty acid composition of plasma membrane leading to the enhanced ethanol tolerance of this strain, were also efficacious to increase the percentage of activation of plasma membrane ATPase per unit of ethanol. These data support a close correlation between the ethanol tolerance of this strain and the sensitivity of its plasma membrane ATPase to the *in vivo* ethanol-induced activation.

Key words ethanol tolerance, phospholipid fatty acid composition, plasma membrane ATPase

The proton-pumping ATPase in the plasma membrane of *Saccharomyces cerevisiae* couples ATP hydrolysis, consuming up to 50% of total cellular ATP, to the expulsion of protons across the membrane, thus generating a transmembrane proton electrochemical gradient which drives active transport of nutrients[1, 2]. The plasma membrane ATPase maintains the intracellular pH of *Saccharomyces cerevisiae* between 6.0 and 7.0 even when large variations in extracellular pH occur[3], regulating the activity of some pH-sensitive intracellular enzymes[1]. Near-maximum ATPase activity is rare, because it is energetically expensive to maintain, and activity is usually much lower[1,2]. For instance, when cells of *Saccharomyces cerevisiae* were incubated *in vivo* with glucose, the plasma membrane ATPase activity increased as much as 10-fold[4]. Growth at acid pH also resulted in an increase in ATPase activity, which appeared to constitute a mechanism for internal pH modulation[5]. Furthermore, the enzyme is a major component of the plasma membrane (accounting for 15% ~ 20% of the plasma membrane protein) and seems to play a significant role in maintaining the structural integrity of the cells, as evidenced by the altered cell morphology present in some ATPase mutants[6]. The plasma membrane ATPase is not only essential for, but also rate-limiting to, cell growth of *Saccharomyces cerevisiae*[2].

It has been shown that the plasma membrane ATPase activity of *Saccharomyces cerevisiae* is activated *in vivo* by ethanol at concentrations that affect growth and fermentation rates, which counteracts the deleterious effects of ethanol[7]. However, little is known about a correlation between this enzyme and other components (such as fatty acids) of plasma membrane in the development of ethanol tolerance of yeast. Thus, the aim of the present work is to investigate the impact of alterations in phospholipid fatty acid composition of plasma membrane on plasma membrane ATPase activity of yeast cells, and its re-

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lationship to ethanol tolerance. The data obtained demonstrate for the first time that phospholipid fatty acid composition of plasma membrane can significantly influence sensitivity of yeast plasma membrane ATPase to *in vivo* activation by ethanol and thus ethanol tolerance. The results reported here may also help to understand the interaction between lipids and proteins in plasma membranes in the development of yeast ethanol tolerance, which is highly needed to explore.

1 Materials and Methods

1.1 Strain, media and culture conditions

The strain SPSC used in this work was a self-flocculating fusant of *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae*, which was constructed using protoplast fusion technology and preserved by our laboratory[8,9].

Agar slope medium for culture maintenance contained (g/L): glucose 10, yeast extract 3.85, peptone 3, agar 20. Growth medium for yeast aggregates formation was composed of (g/L): glucose 30, yeast extract 3.85, peptone 3. To form yeast aggregates, a loopful of cells from agar slope were used to inoculate 100 mL volumes of growth media and the cultures were incubated orbitally at 30℃ for 18 h.

The above growth medium was used as a basal medium, to which 0.6 mmol/L of palmitic, linoleic or linolenic acid, and an emulsifier, 0.1% bovine serum albumin defatted by Chen's method[10], were added. Then the 18-h prepared cell aggregates were inoculated into the medium to a final cell density of 50 mg (dry wt)/L and the cultivation was carried out statically at 30℃ for 20 h. Cells were then harvested and inoculated into the growth medium supplemented with increasing concentrations of ethanol (0% ~ 10%, *V/V*) to give a cell density of 100 mg (dry wt)/L and the cultures were incubated orbitally at 30℃ for 18 h.

1.2 Preparation of plasma membrane

Exponential phase cells were harvested, washed twice with distilled water and then twice with 1.2 mol/L sorbitol solution. Cells were converted to spheroplasts essentially as described by Dickinson & Isenberg[11]. Spheroplasts were then harvested by low-speed centrifugation (700 ~ 800 g) and washed twice with 1.2 mol/L sorbitol. The total membrane fraction was obtained as a pellet after osmotic lysis of the spheroplasts in 0.9% NaCl and centrifugation at 29 000 g for 20 min at 4℃. Then, the plasma membrane fraction was further isolated according to the method of Schibeci *et al*[12]. Protein concentrations in the plasma membrane fraction were determined by the method of Bradford using bovine serum albumin as a standard[13].

1.3 Fatty acid analysis

Plasma membranes were put into a chloroform/methanol solution (2:1, *V/V*) and stirred for 2.5 h. The extracts were washed with a 0.88% (*W/V*) KCl solution and allowed to separate overnight. The lower phase was removed, dried on a rotary evaporator and the residue immediately dissolved in a minimum of chloroform. Phospholipids were separated from other lipid classes by thin layer chromatography according to the method of Cartwright *et al*[14]. To determine the fatty acid composition of phospholipid classes, bands were scraped off to prepare fatty acid methyl esters by heating (80℃, 60 min) the silica gel with 14% (*W/V*) BF_3/methanol solution. The fatty acid methyl esters were analyzed by gas chromatography (Agilent 6890A GC, USA, equipped with flame ionization detector, injector temperature 270℃, detector temperature 250℃).

1.4 Plasma membrane ATPase assay

The ATPase activity of plasma membrane fraction (30 ~ 60 μg of protein per 500 μL of the assay mixture) was determined in a assay medium containing 50 mmol/L MES (pH 5.8, adjusted with Tris), 10 mmol/L $MgSO_4 \cdot 7H_2O$, 50 mmol/L KCl, 5 mmol/L sodium azide, 0.2 mmol/L ammonium molybdate and 100 mmol/L KNO_3. After 6 min of incubation of the assay mixture at 30℃ for the thermostabilization, the enzyme reaction was started by the addition of ATP (final concentration, 2 mmol/L), with a reaction time ranging from 6 to 9 min. Then, the reaction was stopped by mixing the contents with 1.5 mL of a solution containing 2% (*V/V*) H_2SO_4, 0.5% (*W/V*) SDS and 0.5% (*W/V*) ammonium molybdate. The membranes were then separated by centrifugation, and 20 μL of 10% (*W/V*) ascorbic acid was added to the supernatant and the mixture was incubated for 5 min at 30℃. The absorbance of the solution was measured at 750 nm and the value related to Pi concentration by a standard curve. One unit (u) of enzyme activity is defined as the amount of the membrane protein needed to release 1 nmol Pi per min under the conditions described above.

1.5 Characterization of basal and activated plasma membrane ATPases

The kinetic properties of plasma membrane ATPase in the plasma membrane fraction extracted from cells grown with or without ethanol were compared with respect to K_m for ATP, pH profile and sensitivity to orthovanadate (a specific inhibitor of plasma membrane ATPase). The K_m for ATP was calculated by a least-squares fitting to the Lineweaver-Burk plot of ATPase activity versus ATP concentration. The pH profile was drawn in the range of 4.3 to 8.3. To examine the inhibition of plasma membrane ATPase by orthovanadate, the plasma membrane fraction was preincubated for 10 min in an assay medium containing increasing concentrations of $Na_3VO_4 \cdot 14H_2O$ (0 ~ 300 μmol/L) at pH 5.8 and 30℃, and the enzyme reactions were started as described above.

1.6 Effect of ethanol on *in vitro* activity of plasma membrane ATPase

To evaluate the impact of ethanol on the *in vitro* activity of this enzyme, the plasma membrane fraction extracted from cells grown with or without ethanol was incubated in the ATPase assay mixture, at 30℃ and pH 5.8, in the presence of increasing concentrations of ethanol (up to 12%, *V/V*), for 50 min before initiating the enzyme reaction by the addition of ATP.

1.7 Statistical methods

In this work, all experiments were performed at least in duplicate and all determinations were done in triplicate with mean values given.

Table 1 Fatty acid composition of phospholipids in plasma membranes of fusant SPSC grown with or without exogenously supplemented fatty acid[a]

Culture condition[b]	Fatty acid composition/%								Unsaturation index (Δ/mol)[c]
	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3	
1	1.4	3.2	48.6	20.8	8.6	17.3	0	0	0.41
2	3.5	2.6	28.3	17.4	11.5	16.6	20.4	0	0.77
3	2.7	3.0	25.0	15.5	12.0	16.0	0	25.7	1.12
4	3.2	1.6	33.6	26.8	12.0	22.6	0	0	0.51

[a] Fatty acids are denoted by the number of carbon atoms: the number of unsaturated linkages; [b] 1, 2 and 3 represent cells grown with 0.6 mmol/L palmitic acid (16:0), linoleic acid (18:2) or linolenic acid (18:3) respectively while 4 represents cells grown without any of these fatty acids (control); [c] Δ/mol = [1 × (% monoene) + 2 × (% diene) + 3 × (% triene)]/100

2 Results and Discussion

2.1 Effect of phospholipid fatty acid composition of plasma membrane on cell growth activity tolerant to ethanol

As shown in Table 1, when grown with different supplemental fatty acid, such as palmitic, linoleic or linolenic acid, cells were enriched with the kind of added fatty acid in the phospholipid fatty acid composition of the plasma membranes. On the other hand, cells grown in the absence of exogenously supplemented fatty acid (control) had an intermediary content of palmitic acid in the plasma membranes, which was higher than linoleic or linolenic acid-enriched cells but was lower than palmitic acid-enriched cells. Moreover, there was a significant difference in the degree of fatty acid unsaturation among them, which increased with decreasing palmitic acid content in their plasma membranes.

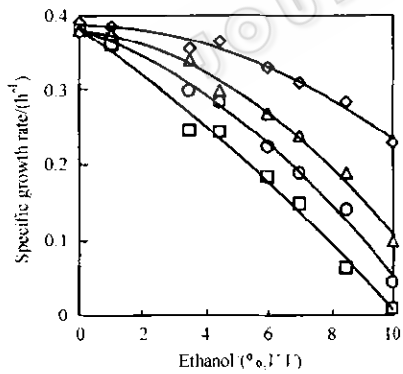

Fig. 1 Maximal specific growth rate of fusant SPSC grown in the presence of increasing concentrations of ethanol

Symbols ◇, ○ and □ represent cells pregrown with 0.6 mmol/L palmitic acid, linoleic acid or linolenic acid respectively, whereas △ represents cells pregrown without any of these fatty acids (control)

Measurement of growth in the presence of ethanol is the frequently employed method for determining ethanol tolerance[15]. Fig. 1 illustrates growth activity tolerant to ethanol of fusant SPSC cells pregrown under different conditions. Although alterations in phospholipid fatty acid composition of plasma membrane did not affect the specific growth rate of cells grown in the absence of ethanol, they markedly affected it when cells were grown with ethanol. At each initial concentration of ethanol (1% ~ 10%, V/V) added to growth medium, the specific growth rate of palmitic acid-enriched cells always remained higher than that of linoleic or linolenic acid-enriched cells or the control. This indicates that an increase in the content of palmitic acid in phospholipid fatty acid composition of plasma membrane results in the enhanced ethanol tolerance of this strain.

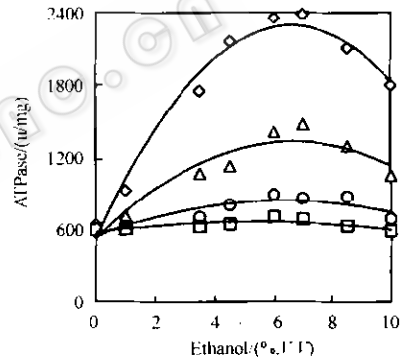

Fig. 2 ATPase activity of plasma membranes extracted from cells of fusant SPSC grown with or without increasing concentrations of ethanol

The definition of each symbol regarding cell pregrowth conditions is identical to that described in Fig. 1

2.2 Effect of phospholipid fatty acid composition of plasma membrane on sensitivity of plasma membrane ATPase to *in vivo* ethanol activation

Fig. 2 shows the plasma membrane ATPase activity of fusant SPSC cells pregrown under different conditions, which were subsequently grown in the absence or presence of increasing concentrations of ethanol. Although alterations in phospholipid fatty acid composition of plasma membranes had no influence on activities of plasma membrane ATPases of cells grown in the absence of ethanol (basal enzymes), they significantly affected the susceptibilities of the enzymes to *in vivo* activation by ethanol: the maximal values for the activated enzymes in cells pregrown with 0.6 mmol/L palmitic acid, linoleic acid, linolenic acid or without fatty acid respectively were 3.6, 1.5, 1.2 and 2.3-fold higher than their respective basal levels (in cells grown without ethanol), with the concentrations of ethanol for the above maximal *in vivo* activation of enzymes being 7%, 6%, 6% and 7% (V/V) respectively. This is the first time to report that phospholipid fatty acid

composition of plasma membrane can markedly alter the sensitivity of yeast plasma membrane ATPase to *in vivo* ethanol-induced activation. Furthermore, the characteristics of phospholipid fatty acid composition of plasma membrane resulting in the increased ethanol tolerance of this strain (Fig. 1), were also effective to enhance the percentage of activation of plasma membrane ATPase per unit of ethanol (Fig. 2). These results support a close relationship between the ethanol tolerance of this strain and the sensitivity of the plasma membrane ATPase to the *in vivo* activation by ethanol.

2.3 Comparison of characteristics of basal and activated plasma membrane ATPases

The above experimental data show that *in vivo* ethanol activation of plasma membrane ATPase occurs in fusant SPSC cells, irrespective of disparities in phospholipid fatty acid composition of plasma membrane. In order to understand the mechanism underlying the *in vivo* ethanol activation of this enzyme, investigation was conducted to compare some characteristics of the basal and the activated plasma membrane ATPases, with the results shown in Fig. 3, 4 and Table 2. For each kind of fusant SPSC cells with different fatty acid composition of plasma membrane, although the V_{max} values differed significantly, the K_m values for ATP, the sensitivities to orthovanadate, and the pH profiles of the basal and the activated enzymes were essentially identical. Moreover, the activated state of enzyme was conserved after the extraction of the plasma membrane fraction despite the reduction or elimination of the ethanol incorporated into the plasma membrane. These suggest that the basal and the activated enzymes might be the same enzyme instead of a modified protein and the presumable stimulation of ATPase biosynthesis caused by ethanol could not be ruled out. In fact, enhancement of the ATP gene transcription via the TUF transcription factor binding to upstream activator sequences, has ever been reported[16].

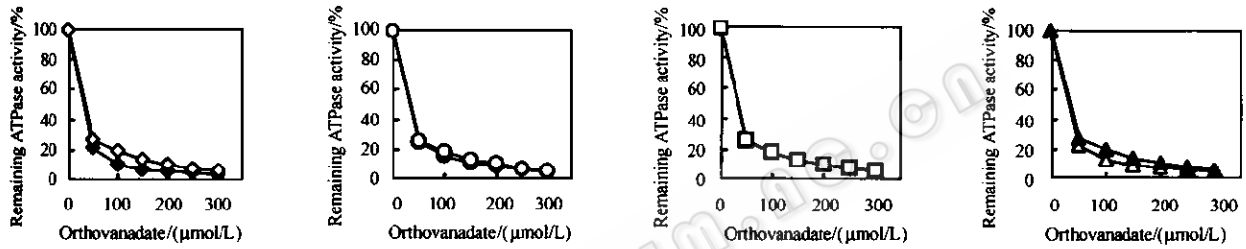

Fig. 3 Effect of orthovanadate on ATPase activity of plasma membranes extracted from cells of fusant SPSC pregrown and grown under different conditions

Remaining ATPase activity (%) = (E/E_0) × 100%, where E and E_0 represent ATPase activity in the plasma membrane fractions which were incubated with or without increasing concentrations of orthovanadate respectively. Symbols representing conditions for cell pregrowth (with or without fatty acid) and growth (with or without ethanol): ◆(16:0, 7%), ◇(16:0, 0%); ●(18:2, 6%), ○(18:2, 0%); ■(18:3, 6%), □(18:3, 0%); ▲(7%), △(0%)

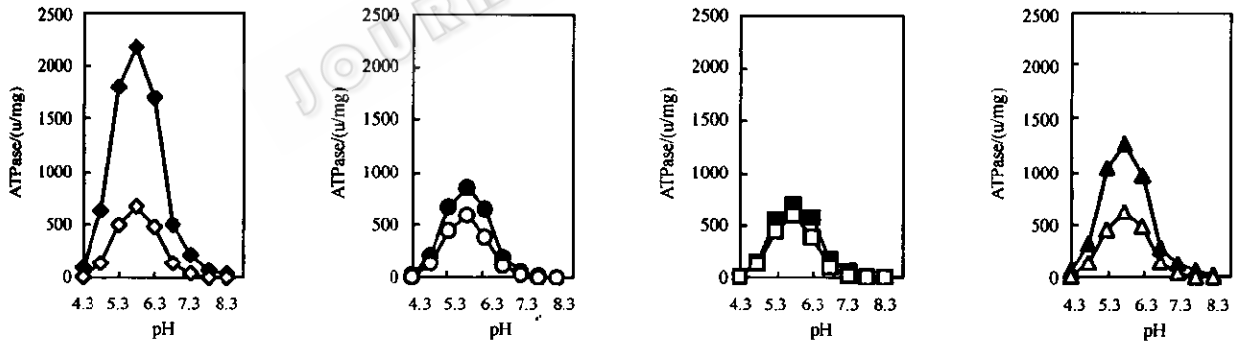

Fig. 4 Effect of pH on ATPase activity of plasma membranes extracted from cells of fusant SPSC pregrown and grown under different conditions

The denotation of each symbol regarding cell pregrowth and growth conditions is identical to that described in Fig. 3

Table 2 Kinetic parameters of plasma membrane ATPase of fusant SPSC pregrown and grown under different culture conditions

Cells pregrown with or without fatty acid*(0.6 mmol/L)	Cells grown with or without ethanol/(%, *V/V*)	K_m/(mmol/L)	V_{max}/(nmol Pi·min^{-1}·mg^{-1})
16:0	Without	0.64	1290
16:0	7	0.60	3900
18:2	Without	0.66	1250
18:2	6	0.63	1990
18:3	Without	0.67	1230
18:3	6	0.63	1830
Without	Without	0.66	1310
Without	7	0.61	2550

*16:0, 18:2 and 18:3 represent palmitic, linoleic and linolenic acids respectively

However, some authors emphasized that, in *Saccharomyces*, the overexpression of plasma membrane ATPase was detrimental for the cell and the regulatory mechanism should be based on modulating catalytic activity and not the amount of the enzyme[17]. Difference observed for some characteristics of basal and activated ATPases induced by acid pH were, in fact, in accordance with this notion[5]. Of course, there might be disparities in the mechanism underlying *in vivo* activation of the enzyme by ethanol or by acid pH. Nevertheless, further investigation is needed in order to understand the precise mechanism involved in the *in vivo* ethanol activation of plasma membrane ATPase of fusant SPSC.

2.4 *In vitro* ethanol inhibition of plasma membrane ATPase

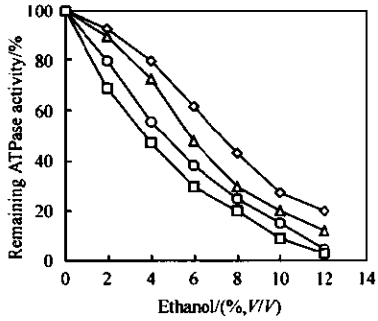

Fig. 5 Inhibition of plasma membrane ATPase by incubation with increasing concentrations of ethanol, of plasma membrane fraction extracted from cells of fusant SPSC grown with 7% (*V/V*) (◇, △) or 6% (*V/V*) (○,□) ethanol

The denotation of each symbol regarding cell pregrowth conditions is identical to that described in Fig. 1. Remaining ATP activity (%) = $(E'/E_0') \times 100\%$, where E' and E'_0 represent ATPase activity in the plasma membrane fractions which were incubated with or without increasing concentrations of ethanol respectively

While activating the plasma membrane ATPase of fusant SPSC *in vivo*, ethanol inhibited the same enzyme *in vitro* (Fig. 5). This result is consistent with that previously reported by Rosa *et al*[7]. Both the basal and the activated ATPases were inhibited by ethanol, and the susceptibilities of the basal ATPases to *in vitro* ethanol inhibition were virtually identical (data not shown). However, a novel phenomenon was observed: the resistance of the activated ATPase to *in vitro* inhibition by ethanol varied with phospholipid fatty acid composition of plasma membrane (Fig. 1 and 5), i.e., the characteristics of fatty acid composition of phospholipids in plasma membranes leading to the increased ethanol tolerance of cells also effectively enhanced the capacity of the enzyme to resist the *in vitro* ethanol inhibition.

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