应用酵母双杂交系统筛选与 CIKS(151-574)相互作用的蛋白质

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摘 要 CIKS(Connection to IKK and SAPK/JNK)是最近发现的细胞蛋白,能激活 IKK 和 SAPK/JNK。应用酵母双杂交系统,将 CIKS(151-574)插入载体 pAS2-1 作为诱饵,筛选人 HeLa 细胞 MATCHMAKER cDNA 文库,以期为阐明 NFκB 及 JNK 活性调控的分子机理提供新的线索。筛选得到 6 个阳性 AD/文库质粒,并用酵母双杂交实验验证了阳性 AD/文库质粒与 CIKS 的相互作用。将阳性 AD/文库质粒测序并对测序结果做 BLAST 分析,发现它们分别是 RIKEN cDNA 473340F03,PLAC8, CD27BP (Siva-1), CDC5L, SnRNP smB,DVL2。CIKS 能与这些功能各异的蛋白质相互作用,表明 CIKS 在细胞的多种生理活动中发挥作用。

关键词 CIKS, HeLa cDNA 文库, 蛋白质相互作用, NF-κB
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NF-κB(Nuclear factor κB)是一类具有重要生理功能的转录因子。因其能调节许多细胞基因的表达而在细胞的炎症反应、免疫反应、细胞增殖、细胞凋亡等生理活动中发挥重要作用。通常情况下,NF-κB 与其抑制蛋白 IκB(Inhibitor of κB)结合而在细胞质内以无活性的形式存在。由于各种因素,如 TNFα(Tumor necrosis factor α)、IL-1(Interleukin-1)、病毒、细菌等因子的刺激,IκB 由 IκB 激酶(IκB kinase, IKK)催化发生磷酸化而迅速被蛋白酶体降解,释放 NF-κB。NF-κB 随即转位至核内,激活相关基因的转录[1]。NF-κB 活性调控的核心是 IKK 活性的调控,多种胞外信号, 如 TNFα、IL-1,经过不同的上游分子的转导后,会聚于 IKK,并最终激活 NF-κB。NF-κB 能被人类免疫缺陷病毒 1 型、人类 T 细胞病毒 1 型、乙型肝炎病毒、丙型肝炎病毒、EB 病毒以及流感病毒等激活[2],研究 NF-κB 活性调控对于阐明这些病毒与宿主细胞的相互关系具有重要意义。

CIKS 是最近发现的由 574 个氨基酸编码的细胞蛋白,在多种组织中均有表达。细胞水平的实验表明,在过表达的情况下,它能激活 IKK 和 SAPK/JNK[3,4]。CIKS 的发现拓展了 NF-κB 和 SAPK/JNK 信号通路相关的蛋白质网络,但对于它与哪些蛋白相关以及它如何与其它蛋白相互作用而发挥其生理功能,目前知之甚少。

酵母双杂交系统作为研究蛋白质相互作用的一种新技术,已被广泛应用于生物学研究的诸多领域。通过酵母双杂交系统筛选得到与已知蛋白质相互作用的蛋白质,它们往往功能上密切相关,共同参与到细胞的生理或病理过程。获得蛋白质相互作用的信息,可以为我们理解细胞及机体生理活动的分子机理提供线索[3]。

我们以缺失 N 端的 CIKS 为诱饵,应用酵母双杂交系统筛选人 HeLa 细胞 cDNA 文库,以期为阐明 CIKS 的功能提供一些线索。

1 材料和方法

1.1 菌种、质粒及文库

大肠杆菌(*E. coli*) DH5α、HB101 均为本室保存。酵母菌 *Saccharomyces cerevisiae* AH109 (*MATa*, *trp1-901*, *leu2-3*, *112*, *ura3-52*, *his3-200*, *gal4Δ*, *gal80Δ*, *LYS2* ::*GAL1UAS-GAL1TATA-HIS3*, *GAL2UAS-GAL2TATA-ADE2*, *URA3* ::*MEL1UAS-MEL1 TATA-*

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lacZ，*MEL1*）菌种由中国医学科学院基础所张晓东博士惠赠。包含 pAS2-1（*GAL4* DNA-BD，*TRP1*，Amp^r）及其它相关试剂的酵母双杂交系统试剂盒及人 HeLa 细胞 MATCHMAKER cDNA 文库（将 cDNA 插入 pGAD GH（*GAL4* AD，*LEU2*，Amp^r）AD 下游）购自 Clontech 公司。pAS2-1/CIKS 由本室构建，人 CIKS cDNA 克隆自子宫 cDNA 文库。限制酶购自 TaKaRa 公司，连接酶购自 Invitrogen 公司，pfu 酶购自上海生工公司。X-α-gal 及 X-β-gal 购自 Clontech 公司。各种氨基酸购自 Sigma 公司。

1.2　pAS2-1/CIKS(151-574)的构建

应用 pfu 酶进行 PCR 反应，以 pAS2-1/CIKS 为模板。上游引物为 5′-CGGAATTCGACACTGGCCATGACTC-3′，下游引物为 5′-CGGGATCCTCACAAGGGAACCACCTGAA-3′。分别在上游引物和下游引物上引入 *Eco*R Ⅰ及 *Bam*H Ⅰ切点。将 PCR 产物 CIKS(151-574)经 *Eco*R Ⅰ和 *Bam*H Ⅰ消化，回收后与经同样条件消化的 pAS2-1 载体连接，并使 CIKS 阅读框与 BD 同框。测序验证。

1.3　酵母感受态的制备、小量转化和大规模文库转化

参照 Clontech 公司提供的酵母双杂交系统说明书。

1.4　人 HeLa 细胞 MATCHMAKER cDNA 文库的扩增

参照 Clontech 公司说明书。用 QIAGEN 公司 Tip2500 柱提取 AD/文库质粒。

1.5　以 pAS2-1/CIKS 为诱饵筛选人 HeLa 细胞 MATCHMAKER cDNA 文库

每次铺皿 50 个，并测定转化效率和转化克隆数以确保筛选文库的规模。将转化子铺 SD(-Leu/-Trp/-His/-Ade/ + X-α-gal)平皿，30°C 培养 4 ~ 5d 后，选取变蓝的克隆并划线至新的 SD(-Leu/-Trp/ + X-α-gal)平皿，30℃培养 4 ~ 5d 后再选取变蓝的克隆画线。反复 3 次后再划线至 SD(-Leu/-Trp/-His/-Ade/ + X-α-gal)平皿，选取仍能变蓝的克隆进行下一步实验。

1.6　AD-文库质粒的回收及分析

按 Clontech 公司的说明书回收酵母中的 AD-文库质粒，并转化大肠杆菌 HB101。按常规方法从细菌中提取 AD-文库质粒。用 AD 载体外源片段插入位点两侧的通用引物扩增插入片段，用 *Hae* Ⅲ分析 PCR 产物。

1.7　pAS2-1/CIKS 诱饵质粒与 AD-文库质粒的特异性双杂交检测

将 pAS2-1/CIKS 与所获得的 AD-文库质粒共转化酵母细胞，检测 lacZ 报告基因活性。

1.8　阳性 AD-文库质粒的序列分析

对阳性 AD-文库质粒采用 AD 通用引物测序，将所得序列信息在 NCBI 网站做 BLAST 分析。

2　结　果

2.1　pAS2-1/CIKS(151-574)诱饵的构建

通过预实验，我们发现用 pAS2-1/CIKS 单独转化酵母细胞就能够激活报告基因的表达，故 CIKS 的全序列不宜用作筛库的诱饵。为此我们构建了 pAS2-1/CIKS(1-420)、pAS2-1/CIKS(151-574)两个缺失突变体并在酵母细胞中检测其激活报告基因的能力，发现 pAS2-1/CIKS(151-574)不能激活报告基因的表达，因此可以作为筛库的诱饵。经测序及酶切鉴定，pAS2-1/CIKS(151-574)的大小及插入位点正确，并与 BD 同框(图 1)。

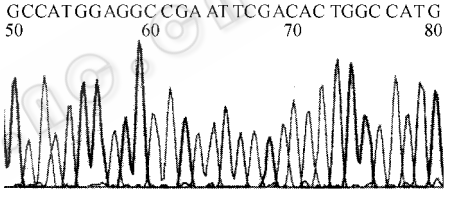

图 1　pAS2-1/CIKS(151-574)测序图

Fig. 1　Sequencing of plasmid pAS2-1/CIKS(151-574)

The junction between CIKS (151-574) and BD is shown. An *Eco*R Ⅰ site (positions 62-67) lies immediately upstream of CIKS (151-574), which fuses in frame to BD

2.2　文库转化及转化子筛选

我们用 150mm × 150 个平皿扩增了 9×10^6 个克隆，并提取 AD/文库质粒用于转化酵母。pAS2-1/CIKS(151-574)与 AD/文库质粒共转化酵母菌 AH109 后，铺皿 50 个，经计算，共筛转化子 3×10^6 个。培养 4 ~ 5d 后，得到变蓝的克隆 46 个。经过 4 轮划线分离后，有 13 个克隆能够变蓝，初步确定为阳性克隆。从阳性克隆中回收 AD/文库质粒，经 PCR 扩增均能得到外源片段(图 2)，为了消除重复的质粒，用 *Hae* Ⅲ消化 PCR 产物，我们发现 5 与 10 酶切指纹相同(图 3)，应为相同的质粒。测序结果也表明它们为相同的质粒。

2.3　pAS2-1/CIKS(151-574)与 AD/文库质粒相互作用的检测

用 pAS2-1/CIKS(151-574)与筛选出的 AD/文库质粒共转化酵母细胞，检测报告基因的活性。与此同时，检测 AD/文库质粒的自激活活性以及与 pAS2-1 共转化的激活活性。结果表明，所有检测的 AD/

文库质粒单独转化酵母时不能激活报告基因的活性，而与 pAS2-1/CIKS(151-574)共转化都能激活报告基因的活性。但是，其中 3、5、10、11 质粒与空的 BD 载体 pAS2-1 共转化酵母时即能激活报告基因的活性。这表明它们是假阳性质粒，而 1、2、4、6、7、8、9、12、13 是阳性质粒(表 1)。

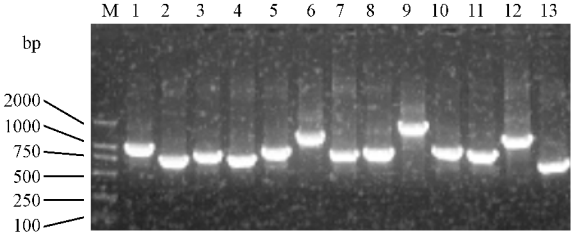

图 2 PCR 鉴定 AD/文库质粒的插入片段

Fig.2 The inserts in the AD/library plasmids were examined by PCR

M. Molecular marker DL2000; 1 ~ 13. Different samples

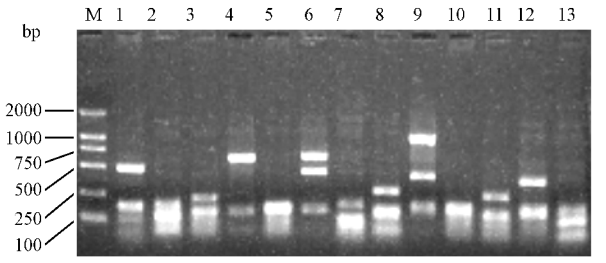

图 3 AD/文库质粒插入片段指纹图(*Hae* Ⅲ酶切)

Fig.3 The fingerprints of inserts in the AD/library plasmids (digested by *Hae* Ⅲ)

M. Molecular marker DL2000; 1 ~ 13. Different samples. The map indicates that No. 5 and No. 10 are identical

2.4 阳性 AD/文库质粒的测序和分析

对阳性 AD/文库质粒测序并做 BLAST 分析结果表明，我们筛选得到的与 CIKS(151-574)相互作用的蛋白质有 RIKEN cDNA 473340F03，PLAC8，CD27BP (Siva-1)，CDC5L，SnRNP smB，DVL2 等(表 2)。

表 1 用酵母双杂交检测 AD/文库质粒与 pAS2-1/CIKS(151-574)的相互作用

Table 1 The interaction of pAS2-1/CIKS(151-574) and AD/library plasmids determined by yeast two-hybrid

AD/library plasmids No.	AD/library plasmids self-activation control	AD/library plasmids cotran-formed with pAS2-1	AD/library plasmids cotran-formed with pAS2-1/CIKS(151-574)
1	–	–	+
2	–	–	+
3	–	+	+
4	–	–	+
5	–	+	+
6	–	–	+
7	–	–	+
8	–	–	+
9	–	–	+
10	–	+	+
11	–	+	+
12	–	–	+
13	–	–	+

表 2 BLAST 结果

Table 2 Result of BLAST

AD/library plasmids No.	GenBank No.	Name of sequence	Function	Homologous region	Identity/%
1	BC013311	RIKEN cDNA 473340F03	Unknown	577 – 998	100
4	NM-016619	PLAC8	Unknown	139 – 573	100
6	NM-001253	CDC5L	Cell cycle regulation, pre-mRNA splicing	1843 – 2301	100
7	M34081	SnRNP smB	Pre-mRNA splicing	310 – 761	100
8	NM-006427	CD27BP(Siva-1)	Inhibits BCL-XL-mediated protection against UV radiation-induced apoptosis	32 – 478	100
9	BC014844	DVL2	Unknown	1159 – 1723	99

3 讨 论

我们以 pAS2-1/CIKS(151-574)为诱饵，应用酵母双杂交系统筛选人 HeLa cDNA 文库得到了能与 CIKS 相互作用的 6 种蛋白质，并在酵母细胞中初步验证了它们的相互作用。其中 CD27BP (Siva-1)，CDC5L，SnRNP smB 已有文献报道其功能，另外 3 种则为功能未知的蛋白质。CD27BP (Siva-1)能与 $BCL\text{-}X_L$ 结合，并抑制 $BCL\text{-}X_L$ 的功能。另外，CD27BP (Siva-1)能调控 T 细胞的内环境稳定[5]。CDC5L 是 mRNA 前体剪接产物形成必需的多蛋白质复合物的核心组分。在过表达的条件下可以缩短细胞周期的 G2 期，缺失 N 端激活结构域的 CDC5L 突变体则通过延滞细胞进入有丝分裂而影响 G2 期。[6]。SnRNP smB 则在 mRNA 前体的剪接中发挥作用[7]。与 CIKS 相互作用的蛋白质涉及细胞生理活动的多个方面，这可能暗示 CIKS 的重要生理作用。有趣的是，CIKS 既能与具有抗凋亡作用的蛋白质(如 IKK)作用，又能与促进细胞死亡的蛋白质(如 CD27BP)作用，是否意味着它参与了细胞生存或死亡的平衡调控。这有待研究。

已有的研究表明，CIKS 能与 IKK 的 3 个亚基相互作用[3]，在我们的实验中，却没有筛选得到编码 IKK3 个亚基的克隆。这可能有两个原因：一方面，我们采用了缺失 N 端的 CIKS 作为诱饵蛋白质，也许 CIKS 与 IKK3 个亚基作用的功能区恰好在此区域，因而不能筛得编码 IKK 各亚基的克隆；另一方面，为了减少假阳性克隆的出现，我们采用了高严谨性(High-stringency)筛选策略，这可能会导致一部分与 CIKS 作用较弱的克隆不能筛出。

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Screening of New Binding Partners of CIKS with Yeast Two-hybrid System

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Abstract　The NF-κB transcription factor plays important regulatory roles in inflammation, apoptosis, immune and stress responses. IκB kinase (IKK) composed of two catalytic subunits and a regulator subunit, acts as a key component of NF-κB activation pathway through phosphorylation of IκB, the inhibitor of NF-κB. CIKS (connection to IKK and SAPK), a newly identified cellular protein, is involved in NF-κB and JNK activation. Although it has been known that CIKS interacts with IKK complex, and activates both IKK and SAPK when overexpressed; the underling mechanisms are poorly understood. To better understand

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the physiological roles of CIKS, we have screened human HeLa MATCHMAKER cDNA library for new binding partners of CIKS by using the yeast two-hybrid system with truncated CIKS (151-574) as the bait. The yeast strain AH109 was sequentially transformed with the bait plasmid and the library. The transformants were screened on SD(-Leu/-Trp/-His/-Ade/ + X-α-gal) selective plates. Positive clones were restreaked on SD(-Leu/-Trp / + X-α-gal) plates three times to allow loss of some of the AD/library plasmids while maintaining selective pressure on both the DNA-BD and AD vectors. After 3 screenings on SD(-Leu/-Trp / + X-α-gal), the positive clones were further verified on SD(-Leu/-Trp/-His/-Ade/ + X-α-gal) plates. The inserts in AD/library plasmids were amplified by PCR and PCR products were characterized by *Hae* Ⅲ digestion to eliminate the duplicates. After screening in selective plates, the positive AD/library plasmids were rescued via transformation of *E. coli* HB101 and the interactions of CIKS (151-574) with positive AD/library plasmids were further assessed by yeast two-hybrid analysis. Finally, the DNA sequences of the positive AD/library plasmids were determined and BLAST analysis against the databases was performed. The BLAST results indicate that the inserts in the positive plasmids encode RIKEN cDNA 473340F03, PLAC8, CD27BP (Siva-1), CDC5L, SnRNP smB, and DVL2. CDC5L is a key component of the multi-protein complex essential for the formation of pre-mRNA splicing product and is not required for spliceosome assembly. A role for CDC5L in the cell division cycle has been precious suggested as its overexpression of this protein in mammalian cells leads to a shortening G2 phase of the cell cycle, and a negative-dominant mutant of CDC5L lacking the N-terminal activation domain delays the G2 phase cell 's entry into the mitosis. It has been reported that SnRNP smB participates in pre-mRNA splicing and CD27BP (Siva-1) binds to and inhibits BCL-XL-mediated protection against UV radiation-induced apoptosis and regulates T cell homeostasis. The functions of RIKEN cDNA 473340F03, PLAC8 and DVL2 are unknown. It has been suggested that CIKS functions as a critical component for cross-talk between NF-κB and JNK signaling pathways. IKK subunits, which have been demonstrated to interact with CIKS, were not shown up in this experiment. We speculate that the truncated CIKS (151-574) bait may not contain the binding domain that mediates the interaction of IKK subunits with CIKS. Taken together, the above results suggest that CIKS may play a role in cell regulation through interacting with various cellular proteins. Further investigations are required to characterize these interactions.

Key words CIKS, HeLa cDNA library, yeast two-hybrid screening, Protein-protein interaction, NF-κB

Biosynthesis of Poly(3-mercaptopropionate) and Poly (3-mercaptopropionate-*co*-3-hydroxybutyrate) with Recombinant *Escherichia coli*

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Abstract Polythioesters newly emerged as a type of novel polymer and they have showed great potential for application in industries. In this study, genes of butyrate kinase (*buk*) and phosphotransbutyrylase (*ptb*) from *Clostridium acetobutylicum*, and poly (3-hydroxybutyrate) (PHB) synthase gene from *Thiocapsa pfennigii* were used for construction of a metabolic pathway to synthesize the polythioesters. When 3-mercaptopropionate and 3-hydroxybutyrate were fed, poly(3-mercaptopropoinate) [poly(3MP)] and poly(3-mercaptopropionate-co-3-hydroxybutyrate) [poly(3MP-*co*-3HB)] were synthesized by recombinant *Escherichia coli* JM109 (pBPP1) harboring the constructed metabolic pathway. Results indicated clearly that all these genes are necessary for the synthesis of poly(3MP) and poly(3MP-*co*-3HB).

Key words poly(3-mercaptopropoinate), biosynthesis, butyrate kinase, phosphotransbutyrylase, poly(hydroxyalkanoic acids)

Sulfur-containing polymers are of interests for industries because of their excellent optical and thermal properties as well as electrical conductivity[1]. Several sulfur-containing polymers such as polythiocarbonates, polythioethers, polythioesters, and polydisulfides have been synthesized chemically[2] and also by plasma polymerization[3]. Only very recently sulfur-containing polyesters originated from biological synthesis were obtained. Takagi *et al* reported the accumulation of polyhydroxyalkanoate with a thiophenoxy side group by *Pseudomonas putida* when 11-thiophenoxy undecanoic acid was used as sole carbon source[4]. Poly (3-mercaptopropionate-*co*-3-hydroxybutyrate) [poly (3MP-*co*-3HB)] was obtained from *Ralstonia eutropha* when 3-mercaptopropionate or 3,3′-thiodipropionate was provided in the cultural media[5]. Up-to-date, this is the only known member of polythioesters that contains thioester bonds in their backbone (Fig. 1). However, the *R. eutropha* could not produce homopolythioester, e.g. poly(3MP), as the enzymes to form the precursors of poly(3HB) could not be depressed in the cells[5-6].

The biosynthetic pathways of these sulfur-containing polyesters were regarded to be identical to the synthetic pathways of polyhydroxyalkanoate (PHA) in the bacteria like *R. eutropha*. The finding that poly(3-hydroxybutyrate) [Poly(3HB)] synthase of *R. eutropha* catalyzed the formation of poly(3MP-*co*-3HB) extended its substrate spectrum and also indicated that this enzyme is capable of catalyzing the formation of a thioester bond. Genetically engineered recombinant *E. coli* strains carrying defined synthetic pathways for accumulating various PHAs exhibit some advantages in synthesizing different PHA molecules. Besides poly(3HB), polyesters like poly (3HB-*co*-3-hydroxyvalerate) [poly (3HB-co-3HV)][7], poly (4-hydroxybutyrate) [poly(4HB)][8], poly(3HB-*co*-4HB)[9] and even terpolymer of 3HB, 4HB and 4-hydroxyvalerate were accumulated in recombinant *E. coli* cells[10]. In this paper, we present that the recombinant *E. coli* JM109 (pBPP1) accumulated poly(3MP) and poly(3MP-*co*-3HB) when 3MP and 3HB were provided. Studies also indicated that the accumulation of these polyesters clearly relied

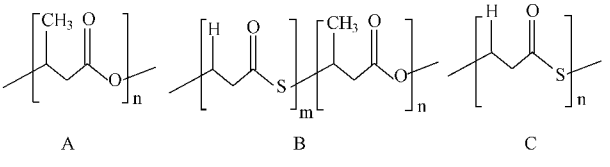

Fig. 1 Structures of poly(3-hydroxybutyrate) (A), poly(3-hydroxybutyrate-co-3-mercaptopropionate) (B), and poly(3-mercaptopropionate) (C)

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on the presence of butyrate kinase, phosphotransbutyrylase and PHB synthase genes.

1 MATERIALS AND METHODS

1.1 Bacterial strains and cultivation

E. coli JM109 harboring pBPP1 was constructed previously[10]. Briefly, the plasmid pBPP1 was created by two steps: First, a 4.4 kb genomic fragment of *Clostridium acetobutylicum* containing the butyrate kinase and phosphotransbutyrylase genes (*buk* and *ptb*) was cloned into pBR322 at its *Bam*H Ⅰ site, resulting plasmid pJC7. Second, a 2.8 kb *Eco*R Ⅰ DNA fragment of *Thiocapsa pfennigii* containing the PHB synthase gene (*pha*EC) was cloned into the pJC7 at its *Eco*R Ⅰ site, resulting plasmid pBPP1. Recombinant *E. coli* cells were cultivated in M9 medium at 37℃ with 1% of glucose and 0.05% of NH_4Cl. 3-MP and 3-HB at different concentrations were supplemented as indicated in the text.

1.2 Effect of 3-MP on growth of *E. coli*

50 mL of M9 medium in a 250-mL flask was inoculated with 0.5 mL pre-culture (cultivated for 24 h in M9 medium) and cultivation was conducted at 37℃, 200 r/min. 3-MP was added to the cultures at indicated times. Growth of cells was monitored with a Kletometer.

1.3 Accumulation of polythioesters

For accumulation of polythioesters, 1 L medium in a 3-L flask was inoculated with 10 mL of precultures. 3-MP was added to the culture at the late exponential growth phase. Cells from 1 L culture were harvested by centrifugation (5000 g for 10 min) and washed with saline once. Centrifugation was repeated and the collected cells were lyophilized. For determination of polyester content in cells, 5 ~ 7 mg lyophilized cells were methanolized in the presence of sulfuric acids, and the resulting methyl esters were determined by GC[11].

1.4 Isolation and analysis of polyesters by gas chromatography and GC/MS

Polythioesters were extracted from lyophilized cells with hot chloroform in a sealed 20 mL glass tube. After 4 h incubation, extracts were filtered and polymers were precipitated in 10 times volume of ethanol. 1 ~ 2 mg of polymer was undergone for methanolysis. 3-MP-methyl ester was identified by GC[11] and GC/MS[5].

1.5 Determination of molecular weight

The molecular weights of purified polyesters were estimated by gel-permeation chromatography (GPC) relative to polystyrene standards. Analysis was performed on four styragel columns connected in line in a Waters GPC apparatus. Samples were eluted with chloroform at a flow rate of 1.0 mL/min and at 35℃, and the eluted compounds were monitored by a Waters 410 differential refractometer.

1.6 IR spectroscopic analysis

The IR spectra of the isolated polymer were recorded with a Fourier transfer spectrometer IFS28 (Bruker). The samples were dissolved in $CHCl_3$ and deposited as a film on a sodium chloride disk. Identification of characteristic adsorptions of samples were performed as described before[5].

2 RESULTS

2.1 Effects of 3-mercaptopropionate on the growth of *E. coli* and accumulation of poly(3MP) in cells

3-MP is toxic to most organisms because of its strong reducing ability and interference to the metabolic reactions in cells. Exposure of 3-mercaptopropionate of concentrations above 0.4% to cells in the cultural broth completely inhibited the growth of *E. coli*. As indicated in Fig. 2, 0.1% of 3-MP affected the growth significantly. However, we found that step-wise feeding to the cultures could reduce the toxic effect on cell growth. The toxic effects of 3-MP on cells could also be minimized after cells had reached high concentrations in cultural broth. If 3-MP was fed after 9 h or 12 h cultivation in M9 media, accumulation of poly(3-MP) occurred (Table 1).

Table 1 Accumulation of poly(3MP) in recombinant *E. coli* JM109/pBPP1*

Cultivations	1	2	3	4
Addition of 3-MP	No addition	0 h	9 h	12 h
Poly(3MP)(% of CDW)	0	0	12.2	16.8

* Cells were cultivated in 300-mL flasks containing 50 mL of M9 media at 37℃, 200 r/min, for 60 h. 0.1% of 3-MP and 0.4% of 3-MP were added to cultures as indicated in the Table. Lyophilized cells were analyzed by GC.

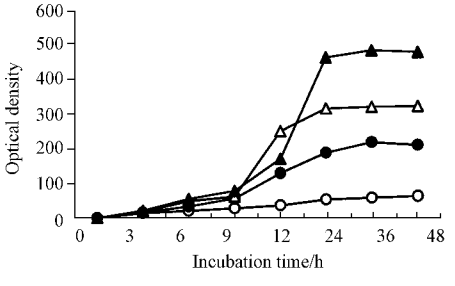

Fig.2 Effect of 3-mercaptopropionate on the growth of *E. coli*
○. 0.1% of 3-MP at the beginning; ●. No addition of 3-MP; △. 0.2% of 3-MP after 9 h cultivation; ▲. 0.2% of 3-MP affter 12 h cultivation

2.2 Buk, Ptb and PhaEC genes are required for incorporating 3-MP into polymers

In order to confirm that the accumulation of poly(3MP) was initiated by Buk, Ptb and PHB synthase, and to confirm the synthetic pathway of Poly(3MP), *E. coli* cells containing the respective genes on plasmids were examined for the accumulation of poly(3MP). Results (Table 2) clearly indicated that all the three genes were necessary for the synthesis of poly(3MP). This indicated that the synthetic pathway of poly(3MP) were the same as described previously[10] (Fig. 3).

Table 2 Accumulation of poly(3MP) in *E. coli* cells harboring different plasmids*

Strains	Poly(3MP) content (% of CDW)
JM109/pJC7	0
JM109/pPhaEC	0
JM109/pBPP1	10.2

* Cells were cultivated in 300 mL flasks containing 50 mL of M9 media at 37℃, 200 r/min, for 60 h. 0.2% of 3-MP were added to cultures after 12 h pre-cultivation. Lyophilized cells were subjected to GC analysis. pJC7, pPhaEC, and pBPP1 contain the *buk* and *ptb*, PHB synthase genes, and all the three genes, respectively.

2.3 Accumulation of homo-and hetero-polythioesters

During experiments we found that cells accumulated various polythioesters depending on differently feeding strategies. When only 3-MP was fed at the exponential stage of growth, poly(3MP) was accumulated in cells. When 3-MP and 3-HB were co-fed at the exponential stage of growth, heteropolyesters of 3-MP and 3-HB were accumulated in cells (Table 3). Moreover, the molar content of the synthesized heteropolymers depended largely on the ratio of 3-MP to 3-HB applied to the cultures (Table 4).

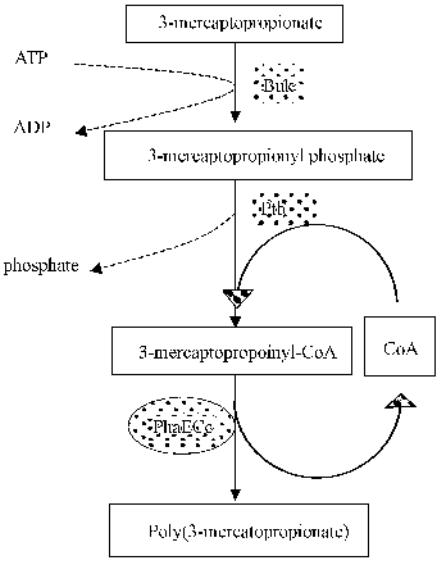

Fig.3 Engineered pathway for biosynthesis of poly(3MP)

Table 3 Accumulation of homo-and hetero-polythioesters in *E. coli* cells*

No.	Substrates and feeding times							Polymer content/%	Molar ratioes (3-MP:3-HB)
	0 h		16 h		40 h		65 h		
	3-MP	3-HB	3-MP	3-HB	3-MP	3-HB	3-MP		
1	–	0.01%	–	0.01%	–	–	–	15.8	3-HB only
2	0.1%	–	0.01%	–	–	0.01%	–	6.7	3-MP only
3	–	0.01%	0.1%	0.01%	0.1%	0.01%	0.1%	24.3	1/3
4	–	0.01%	0.1%	–	0.1%	–	0.1%	18.6	18/1

* The cultural conditions were the same as Table 1. Exception is the addition of substrates indicated in the table. "–": No feeding.

Table 4 Synthesis of heteropolythioesters of various ratios of 3-MP to 3-HB*

	Substrates		Cell yield/(g CDW/L)	Polymer contents			3MP:3HB	Mw
	3HB/%	3MP/%		Total/%	3MP/%	3HB/%		
1	0.05	0.1	0.43	18.5	10.1	8.4	1.2:1	8.5×10^5
2	0.05	0.2	0.34	16.0	12.4	3.6	3.3:1	1.1×10^4
3	0.05	0.4	0.13	15.5	13.6	1.9	6.9:1	ND
4	0.05	0.6	0.10	16.3	14.8	1.5	9.5:1	ND
5	0.05	0.8	0.10	10.0	8.9	1.1	7.8:1	ND

* Cultivations were conducted in 300 mL baffled flasks. R-3HB and 3-MP at indicated concentrations (Table 4) were added after 10 h cultivation. Cells were harvested after 48 h cultivation.

2.4 Characterization of polythioesters by GC-MS, IR and GPC

GC analysis of cells cultivated in M9 media and supplemented with 3-MP at exponential growth stage indicated a peak with retention time of RT = 8.54 min. When both 3-MP and 3-HB were supplemented to the cultures, an additional peak at RT = 9.14 min that was identified previously as 3-HB methyl ester, appeared (Fig. 4). The polymer was isolated from the cells and purified by repeated precipitations in ethanol. Purified polymer was subjected to methanolysis and GC-MS analysis. The peak at RT = 8.54 min was identified to be 3-MP-methyl ester (Fig. 5 A and B). In addition, S-methylmercaptopropionic acid methyl ester was also detected, as indicated by the peak at retention time of 10.55 min (Fig. 5A). Molecular weights of two copolymers were determined by GPC to be 8.5×10^5 and 1.1×10^4. It was found that the molecular weights of heteropolymers decreased as the 3MP ingredients increased (Table 4).

The IR spectrum of poly(3MP) confirmed the thioester bond linkage of the C = O group at 1674 cm^{-1}. Besides the adsorption at this position, a second adsorption at 1408 cm^{-1} appeared and this band need to be identified (Fig. 6).

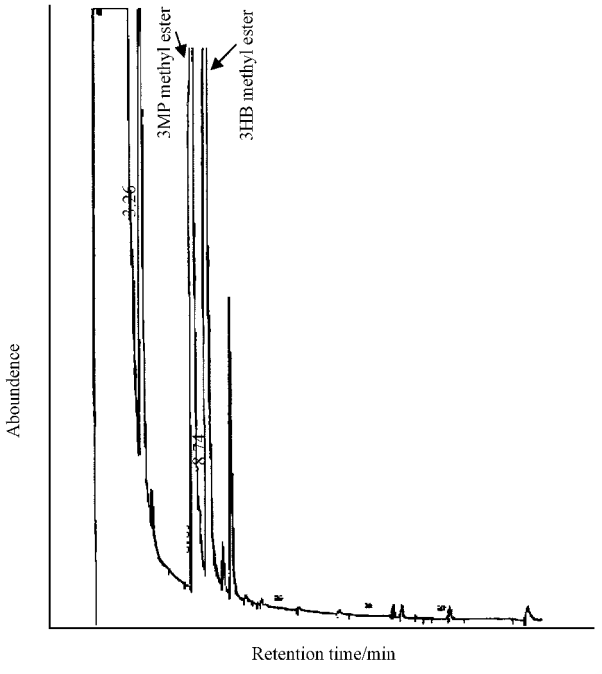

Fig.4 Detection of 3-mercaptopropionate methyl ester and 3-hydroxybutyrate methyl ester by GC

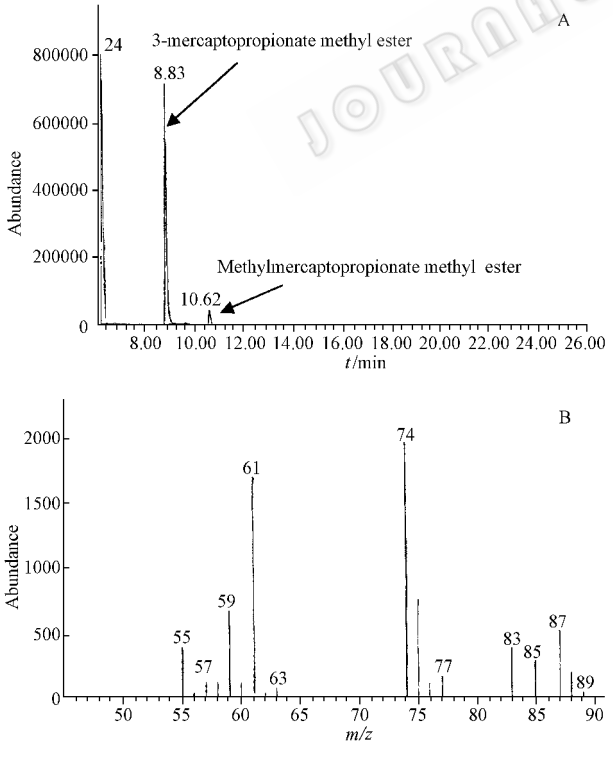

Fig.5 GC/MS analysis of poly(3MP) from recombinant *E. coli*
The purified polymer was hydrolyzed by methanol in the presence of sulfuric acid, and the methyl esters were separated by GC (A). The mass spectrum of the 3-MP methyl ester is presented (B)

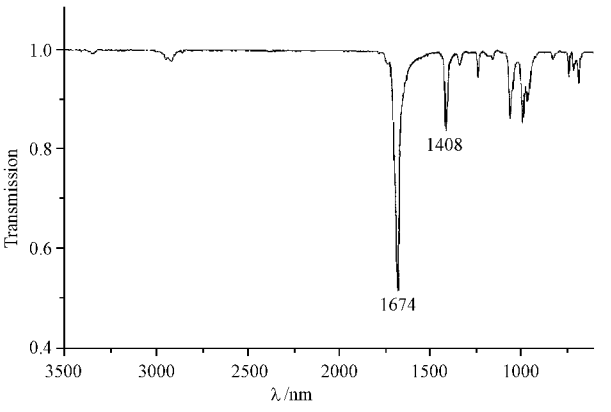

Fig.6 IR spectrum of poly(3MP) from recombinant *E. coli*

3 DISCUSSION

For the first time, a genetically engineered pathway for biosynthesis of 3MP-containing polyesters has been established in *E. coli*. Besides poly(3MP-*co*-3HB), homopolyester of 3-MP, i.e., poly(3MP) was also synthesized by this recombinant *E. coli*. This study indicated that the enzymes, butyrate kinase, phosphotransbutyrylase of *C. acetobutylicum*, and PHB synthase of *T. pfennigii* took 3-MP, 3MP-phosphate, and 3MP-CoA ester as substrates, respectively. Considering the previous finding that PHB synthase of *R. eutropha* catalyses the formation of 3MP-containing polyesters, we conclude that PHA synthases are more catalytically versatile and can initiate the formation of both oxoester and thioester bonds of polyesters.

Homopolythioesters may have unique properties and meet specific requirements to materials. We found that poly(3MP) are difficult to be extracted from lyophilized cells by hot chloroform, compared to poly(3HB) or poly(3HB-*co*-3-HV). The extracted polyester had very limited solubility in chloroform or acetone. Their chemical and physical properties have been recently characterized[6]. The potential applications of poly(3-MP) are needed further to be revealed and evaluated, and large amounts of polymers are needed for such studies and evaluations. The ready-to-use technologies of *E. coli* fermentation would be great help for obtaining large quantity of polythioesters from recombinant *E. coli* cells, as having been proved in an independent publication[6].

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