KOR. JOUR. MICROBIOL June, 1994 p. 192~197 Vol. 32, No. 3

Evidence that a Plasmid Encodes Genes for Metabolism of Malonte in *Pseudomonas fluorescens*

Kim, Yu Sam* and Eun Joo Kim

Department of Biochemistry, College of Science Bioproducts Research Center, Yonsei University, Seoul 120-749, Korea

Pseudomonas fluorescens **which is able to utilize malonate as a sole carbon source was found to contain a novel 60 kb plasmid, which encodes the genes for the proteins to assimilate malonate, including malonate decarboxylase and acetyl-CoA synthetase. The evidence is as follows: The *Pseudomonas* cured with mitomycin C was unable to grow on malonate-medium as well as it lost plasmid. The plasmid isolated from the *Pseudomonas* could be introduced into *E. coli* strain JM103 and DH1 by transformation. The transformed *E. coli* was able to grow on malonate-medium and could transmit its plasmid back to the cured *P. fluorescens* by conjugation. The existence of the plasmid in the transformed *E. coli* was confirmed by hybridization with a labeled probe prepared from 12 kb segment of the plasmid. Dot hybridization showed that the copy number of the plasmid in the transformed *E. coli* is at least 13 times higher than in the wild type *P. fluorescens*. The two key enzymes, malonate decarboxylase and acetyl-CoA synthetase, were inducible by malonate in the transformed *E. coli*.**

KEY WORDS □ **malonate, plasmid, *Pseudomonas fluorescens***

Malonate, which has been studied extensively as a competitive inhibitor of succinate dehydrogenase, has long been known to occur naturally in legumes (17) and in rat brain (10). However, little is known about its metabolism and physiological role. It has been suggested that malonate in soybean seedling root tissue is formed from malonyl-CoA (4). The fate of malonate in the eucaryotic cell has not yet been clearly elucidated, although Hayaishi (4) suggested a metabolic conversion of malonate to malonyl-CoA in the presence of CoA and ATP in the bacteria, malonate adapted *Pseudomonas*. However, this proposition has been contradicted by the isolation of two key enzymes, malonate decarboxylase and acetyl-CoA synthetase in *P. fluorescens* (not published).

It is not clear yet how many gene products are involved in the first stage of assimilation of malonate and how the genes are organized in *P. fluorescens*.

In this report, we present the evidence that there is a plasmid carrying genes encoding for proteins involved in the assimilation of malonate in *P. fluorescens*.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 1.

Growth of bacteria

Pseudomonas fluorescens ATCC 11250 was grown on a medium, pH 6.8, containing 0.6%(w/v) malonate, 0.3% NH_4Cl, 0.2% K_2HPO_4, 0.04% $MgSO_4$, and 0.001% $FeSO_4$, at 30 °C. *E. coli* was grown on LB medium at 37 °C. The transformed *E. coli* was grown in the same medium as *P. fluorescens*, but at 37 °C.

Curing

P. fluorescens was inoculated into 2 m*l* of LB media containing 0, 5, 10, 15, and 20 μg/m*l* of mitomycin C, respectively and cultivated at 30 °C for 48 h with vigorous shaking. The cells in each culture were diluted with 10 μmM $MgCl_2$ to about 2000 cells/m*l*. 100 μ*l* aliquots of the culture were spread on each nutrient agar plate and the plates were incubated at 30 °C for 12 h. By using toothpicks, the individual bacterial colonies were transferred onto the nutrient agar plates and the malonate agar plates. *P. fluorescens* which were unable to grow on malonate agar plates were selected.

Plasmid DNA from *P. fluorescens* was isolated by a modification of the procedure of Maniatis (13). Cells grown to late logarithmic phase were harvested, washed with 100 m*l* STE buffer(0.1 M NaCl, 10 mM Tris HCl, pH 8.0, and 1 mM

Table 1. *Bacterial strains and plasmids used in this study.*

Strains and plasmids	Characteristics	References
Strains		
Pseudomonas fluorescens ATCC11250	Original host of pPSF1	
E. coli		
DH1	F$^-$, *recA1, endA1, gyrA96 thi*$^{-1}$, *hsdR17, supE44*	(3)
JM103	*thi*$^-$, *strA, supE, endA, sbcB, hsdR,1* *F'traD36, proAB, lacI*^{q}, lacZ, M15	(3)
Plasmids		
pPSF1	Ap^r, Km^r, Sm^r	This work
RK2	Ap^r, Km^r, Tc^r	(19)

Ap, Ampicillin; Km, Kanamycin; Tc, Tetracyclin; Sm, Streptomycin.

EDTA), and resuspended in 10 m*l* ST buffer (25% sucrose and 50 mM Tris HCl, pH 8.0). Then 8 m*l* of 0.25 M EDTA, pH 8.0, was added to the cell suspension and the mixture was incubated on ice for 10 min. 4 m*l* of 10% SDS solution was added and mixed with a gentle inversion (20 times per minute) to prevent shearing of the plasmid DNA. As soon as mixing was completed, 6 m*l* of 5 M NaCl was added and the mixture was stored on ice overnight. After centrifugation at 25,000×*g* for 30 min, 1.25 volume of isopropanol was added to the decanted supernatant and the mixture was kept at −20 °C for 2 h. The supernatant was discarded and the pellet was resuspended with 6 m*l* TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0). Plasmid DNA was purified from this DNA preparation by CsCl density gradient centrifugation. Plasmid DNA from the transformed *E. coli* was prepared by the method of Birnboim and Doly (1).

Determination of antibiotic resistance

Two methods were applied for the estimation of antibiotic resistance. LB agar plates containing antibiotics in the manner of concentration gradient (0 to 500 μg/m*l*) were prepared according to Nester (15). The bacteria to be tested were spread on these plates and incubated at 30 °C for 12 h. Secondly, the bacteria were inoculated into LB media, each containing separately the antibiotics, ampicillin (100 μg/m*l*), kanamycin (50 μg/g/m*l*), gentamycin (50 μg/g/m*l*), streptomycin (50 μg/g/m*l*), and tetracycline (5 μg/g/m*l*), and incubated at 30 °C for 48 h with vigorous shaking. After incubation, the turbidity of the culture at 600 nm was measured to observe the effect of the antibiotics.

Transformation of *E. coli* with plasmid pPSF1

Plasmid pPSF1 isolated from *P. fluorescens* ATCC 11250 was introduced into *E. coli* as described previously.

Plasmid transmission by conjugation

Conjugation was carried out by mixing broth cultures of the transformed *E. coli* as a donor and *P. fluorescens*, which was cured and not able to utilize malonate, as acceptor. The mating mixture was incubated for 3 h at 30 °C without shaking. Selection of the exconjugant *P. fluorescens* was performed by using an inositol agar plate (15) containing 1% inositol, 0.3% NH_4Cl, 0.2% KH_2PO_4, 0.04% $MgSO_4$, 0.001% $FeSO_4$, and 2% bactoagar at 30 °C. The other experiment was carried out with *P. fluorescens* as donor and *E. coli* as acceptor. In this case, selection was performed by using the malonate plate described above, incubated at 37 °C.

Enzyme assay

Malonate decarboxylase activity was determined by measuring CO_2 formation from malonate. The assay mixture contained the following components: 50 mM MOPS, pH 7.0, 2 mM malonate containing 0.038 μg/Ci ^{14}C-malonate (1 : 100=^{14}C-malonate: malonate) and an appropriate amount of malonate decarboxylase in a total volume of 200 μg/*l*. CO_2 was captured on Whatman filter paper damped with 2 N KOH and radiolabelled CO_2 was measured by scintillation counter.

Since *E. coli* has its own acetyl-CoA synthetase, direct comparison between *E. coli* and transformed *E. coli* is inadequate. Thus, acetyl-CoA synthetase activity was determined indirectly. This assay is based on the results of unpublished experiments that show that acetyl-CoA synthetase and malonate decarboxylase are coupled during reaction. Assay mixture contained (in micromoles): potassium phosphate buffer (pH 7.2) 100, magnesium chloride 20, sodium malonate 4, ATP 0.1, CoA 0.1, and enzyme (final volume 1 m*l*). The rate of increase in absorbance at 232 nm was recorded by a spectrophotometer. This method (8) is based on the measurement of the increase in absorbance at 232 nm by the formation of the thioester bond of acetyl-CoA following malonate decarboxylation. Protein concentration was determined by the method of Smith (16) with bovine serum albumin as standard protein.

Immunodiffusion

The acetyl-CoA synthetase from *P. fluorescens* was purified by ammonium sulfate fractionation,

Table 2. *Curing efficiency.*

Mitomycin C (mg/ml)	Malonate colony* (%)
0	0
5	8
10	10
15	17
20	19

* Colonies which are unable to grow on malonate plate.

Sephacryl S-300 gelfiltration chromatography, and DEAE Sephacel anion exchange chromatography. Antibody was prepared against acetyl-CoA synthetase in rabbit and it was partially purified by ammonium sulfate precipitation and ion exchange chromatography with DEAE Sephacel.

Immunodouble diffusion was performed by the method of Ouchterlony (2). DNA fragment of about 12 kb was prepared by the digestion of pPSF1 with *Kpn*I and the fragment was subsequently separated by electrophoresis, followed by spin elution (5). The DNA fragment labeled with [α-^{32}P] dATP or dCTP by nick translation to a specific activity of 2×10^7 cpm/μg/g DNA was used as a probe for dot blotting. Bacteria (10^9 cells) grown overnight were harvested by a microcentrifuge and resuspended in 500 m*l* of PBS. Aliquots of this cell suspension (50 m*l*, 5 m*l*) were applied to nitrocellulose filter. The filters were treated by the method of Maniatis (13) for hybridization. Hybridization was performed in the solution containing 50% formamide at 42 °C for 24 h.

RESULTS

Loss of the ability for malonate assimilation by curing

P. fluorescens ATCC 11250 was able to utilize malonate as a sole carbon source. However, upon treatment with mitomycin C, some bacteria were unable to grow on malonate medium. As shown in Table 2, when the bacteria were treated with mitomycin C at a concentration of 5 μg/g per m*l*, 8 out of 100 colonies were unable to utilize malonate.

Isolation of a plasmid from *P. fluorescens* ATCC 11250 and transformed *E. coli*

A plasmid (designated as pPSF1) was isolated from *P. fluorescens* grown on malonate as the sole carbon source. The plasmid was compared by gel electrophoresis with *Klebsiella* plasmid, RK2(60 kb) (18) (Fig. 1). Digestion by restriction endonuclease and subsequent gel electrophoresis revealed the plasmid to be about 60 kb (Fig. 2).

Determination of antibiotics resistance

P. fluorescens and transformed *E. coli*, but not

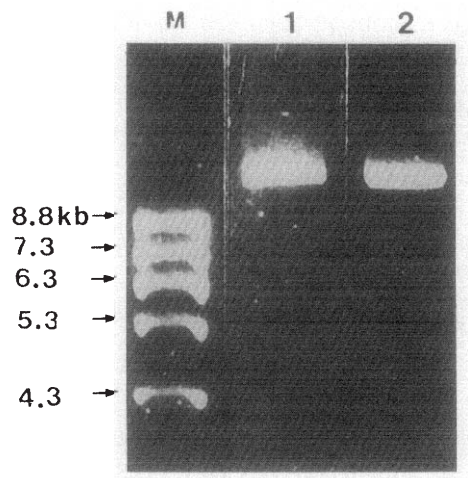

Fig. 1. *Agarose gel electrophoresis of plasmid DNA preparations from P. fluorescens to the E. coli strains.*
M, Marker, SP6 DNA digested with *Hin*dIII; Lane 1, pPSF1; 2, RK2.

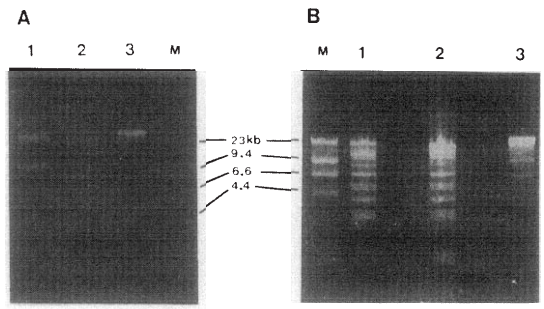

Fig. 2. *Digestion of plasmid pPSF1 with several restriction endonucleases.*
M, lambda DNA; Lane 1, *Pst*I+*Kpn*I; 2, *Pst*I+*Bam*HI; 3, *Kpn*I+*Bam*HI.

the cured *P. fluorescens*, could grow to saturation state on the media containing antibiotics, such as ampicillin(100 μg/g/m*l*), kanamycin(50 μg/g/m*l*), and streptomycin(50 μg/g/m*l*). But on the media containing gentamycine and tetracycline, any bacteria described above did not show O.D. increase. These results indicated that the plasmid contained genes resistant to these antibiotics.

Introduction of pPSF1 into *E. coli* and the cured *P. fluorescens* by transformation and by conjugation

The plasmid could be introduced into *E. coli* DH1 and JM103, which does not contain plasmid (3), by a conventional transformation procedure. Although the *E. coli* strain DH1 and JM103 are unable to utilize malonate, the transformed *E. coli* DH1 and JM103 were able to grow on an agar

Table 3. *Conjugation efficiency (colonies/donor cell).*

	DNA recipient	
	E. coli DH1	Cured *P. fluorescens*
Selection marker	Malonate$^+$	Inositol$^+$
DNA donor		
P. fluorescens	0.19×10^{-6}	
Transformed		
E. coli DH1		2.97×10^{-6}

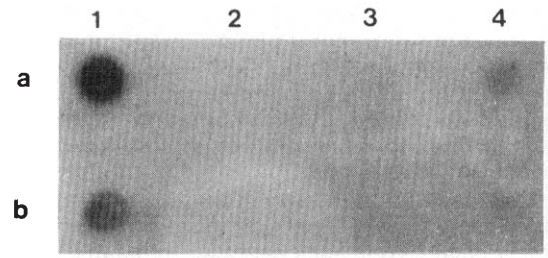

Fig. 3. *Autoradiogram of plasmid DNA hybridized with ^{32}P labeled probe prepared by extracting 12 kb KpnI fragment from pPSF1.*
Cells lysed on nitrocellulose filters were hybridized with the nicktranslated probe.
1, transformed *E. coli*; 2, DH1; 3, cured *P. fluorescens*; 4, *P. fluorescens*.

plate containing malonate as a sole carbon source. The transformation rates were $10^{-7}\sim10^{-6}$ colonies/ recipient cell/ g of plasmid DNA. In order to ensure that the transformed strain was still *E. coli* rather than a contaminant, TSI cell test, which confirmed the identity, was performed (12).

The plasmid could also be introduced from the transformed *E. coli* into the cured *P. fluorescens*. Conjugation efficiency is shown in Table 3. Since only *P. fluorescens* (7) but not transformed *E. coli* and cured *P. fluorescens* (this work) could grow on inositol plate, inositol plate could be used as a selection media. The selected *P. fluorescens* recovered the ability to utilize malonate as a carbon source. As shown in Table 4, bacteria containing the plasmid were able to grow on malonate media and contained acetyl-CoA synthetase and malonate decarboxylase activity. These results suggest that the plasmid carries genes encoding proteins for malonate assimilation, including genes for the two enzymes, malonate decarboxylase and acetyl-CoA synthetase, and several resistant genes against the antibiotics described above, and that the plasmid is transmissible between *P. fluorescens* and *E. coli*. The introduction of the plasmid into the competent *E. coli* was also confirmed by dot

Table 4. *Malonate decarboxylase and acetyl-CoA synthetase activities in the crude extracts from P. fluorescesns and E. coli strains.*

Strain	Specific activity (μmole/min/mg)	
	Malonate decarboxylase	Acetyl-CoA synthetase
P. fluorescens	7.17	11.7
E. coli DH1	N.D	N.D
Transformed *E. coli*	16.3	12.3

N.D., not detected.

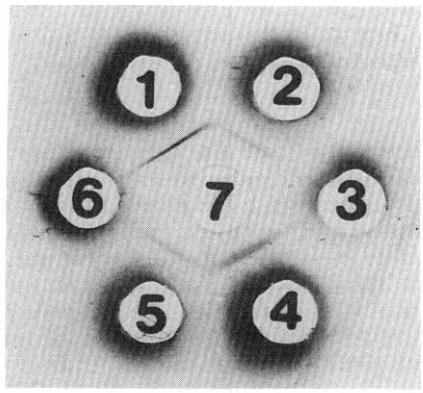

Fig. 4. *Ouchterlony double immunodiffusion.*
Crude cell free extracts grown on malonate. 1,4, *P. fluorescens*; 2,5, transformed *E. coli*; 3, DH1; 6, cured *P. fluorescens*; center well, 7, was filled with rabbit anti acetyl-CoA synthetase antibody.

hybridization with a labeled DNA probe prepared from one segment of the plasmid (Fig. 3).

Assay of the two key enzymes from the transformed *E. coli* and immunological comparison with that from *P. fluorescens*

When the transformed *E. coli* was grown on malonate, the two key enzymes of the proposed pathway for the assimilation of malonate could be detected with sufficient specific activities to explain the growth (Table 4). Since *E. coli* DH1 contains acetyl-CoA synthetase gene, it was important to identify whether the induced acetyl-CoA synthetase is a plasmid coded gene product. The identity of the enzyme from *Pseudomonas* was determined by Ouchterlony (2) double immunodiffusion experiment. As shown in Fig. 4, a fused precipitin line between the enzymes and antibody raised against *P. fluorescens* acetyl-CoA synthetase formed. However, the antibody-precipitable enzymes were not detected in *P. fluorescens* or transformed *E. coli* when they were grown on LB medium. Western blot analysis also

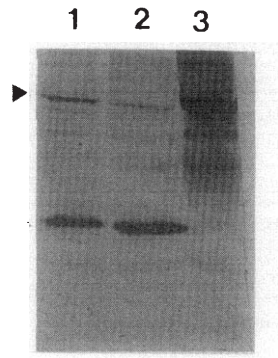

Fig. 5. *Western blot on nitrocellulose treated with rabbit anti acetyl-CoA synthetase antibody.* Crude extracts from lane 1, *P. fluorescens*; 2, transformed *E. coli*; 3, DH1.

showed that the same size acetyl-CoA synthetase was induced in malonate-grown *Pseudomonas* and in malonate-grown transformed *E. coli* (Fig 5). The positive band appeared in 30 kD protein was believed to be a fragment of the enzyme.

These results clearly show that the genes for the proteins required for the assimilation of malonate are encoded by a novel transmissible plasmid, pPSF1, in *P. fluorescens*.

DISCUSSION

Studies on malonate have drawn more attention recently since malonate may play some role in brain development (11) and in symbiotic nitrogen metabolism (9). It has been reported that, in rat brain mitochondria, malonate may play a role in the regulation of the conversion of glutamate to aspartate by inhibiting succinate dehydrogenase and malate transport. In the symbiosis between *Rhizobium japonicum* and soybean, malonate has been proposed to be involved in the nitrogen flow from bacteroids to the plant cell through the malonamate shuttle. It is known that the metabolic conversion of malonate in the brain involves its activation to malonyl-CoA which is then decarboxylated to acetyl-CoA. However, in malonate-adapted *P. fluorescens*, conversion involves malonate decarboxylation to acetate which is then activated to acetyl-CoA. In this pathway, two key enzymes, acetyl-CoA synthetase and malonate decarboxylase are involved. However, little is known about the genes involved in the pathway. There have been several studies on the ability of various TOL plasmids to be cured by growth on benzoate minimal medium (19). The common feature of TOL plasmid appears to be its possession of genes encoding for the enzymes involved in the dissimilation of m- and p- toluate and benzoate. Some *Pseudomonas* hosts of the TOL plasmids possess an alternative chromosomally-encoded pathway for benzoate metabolism. Whereas the plasmid pathway is induced by benzoate, the chromosomal pathway is induced by one of the intermediates of benzoate metabolism. For malonate assimilation, it is highly unlikely that there is an alternative chromosomal metabolic pathway since all intermediates are normal metabolites. It is shown here that in *P. fluorescens* which is able to utilize malonate as a sole carbon source, a transmissible plasmid encodes genes for proteins, including malonate decarboxylase and acetyl-CoA synthetase, involved in malonate assimilation. This novel plasmid is similar in size to but distinct from *Pseudomonas* plasmids reported previously in its possession of genes that are resistant against antibiotics (18). There are similar pathways to that of *P. fluorescens* for malonate metabolism, involving decarboxylation of malonate to acetate, in *Malonomonas rubra* (6). Other pathways, involving the formation of malonamate from malonate and ammonia, takes place in *Rhizobium japonicum* (9). In these bacteria, the plasmid encoded genes for assimilation of malonate has not yet been reported.

All the evidence described above clearly shows, for the first time, that the plasmid, pPSF1, encode genes for assimilation of malonate in *P. fluorescens*. The characterization of the plasmid is under way.

ACKNOWLEDGEMENT

This work was supported by a Genetic Engineering Research grant from the Ministry of Education.

REFERENCES

1. **Birnboim, H.C. and J. Doly,** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* **7**, 1513-1523.
2. **Catty, D. and C. Kaykundalia,** 1988. Antibodies: A practical approach, Vol 1, p. 140-145. IRL Press, Oxford.
3. **Hanahan, D.,** 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* **166**, 557-580.
4. **Hayaish, O.,** 1955. Enzymatic decarboxylation of malonic acid. *J. Biol. Chem.* **215**, 125-136.
5. **Heery, D.M., F. Gannon, and R. Powell,** 1990. A simple method for subcloning DNA fragments from gel slices. *Trends Genet.* **6**, 173.
6. **Hilbi, H., I. Dehning, B. Schink, and P. Dimroth,** 1992. Malonate decarboxylase of *Malonomonas rubra*, a novel type of biotin-containing acetyl enzyme. *Eur. J. Biochem.* **207**, 117-123.

7. **Ingraham, L.C., M.H. Richmond, and R.B. Sykes,** 1973. Molecular characterization of the R factors implicated in the carbenicillin resistance of *Pseudomonas aeruginosa* strains isolated from burns. *Antimicrob. Agents. Chemother.* **3**, 279-288.
8. **Kim, Y.S. and S.K. Bang,** 1988. Assay of malonyl-CoA synthetase. *Anal. Biochem.* **179**, 45-49.
9. **Kim, Y.S. and H.Z. Chae,** 1990. A model of nitrogen flow by malonamate in *Rhizobium japonicum*-soybean symbiosis. *Biochem. Biophys. Res. Comm.* **169**, 692-699.
10. **Koeppen, A.H., E.J. Mitzen, and A.A. Ammoumi,** 1974. Malonate metabolism in rat brain mitochondria. *Biochemistry* **13**, 3589-3595.
11. **Koeppen, A.H. and K.M. Riley,** 1987. Effect of free malonate on the utilization of glutamate by rat brain mitochondria. *J. Neurochem.* **48**, 1509-1515.
12. **MacFaddin, J.F.,** 1980. Biochemical tests for identification of medical bacteria, p. 190-194. Williams & Wilkins, Baltimore.
13. **Maniatis, Y., J. Sambrook, and E.F. Fritsch,** 1989. Molecular cloning: A laboratory manual, p. 1.25-1.41. Cold Spring Harbor Laboratory, Cold Spring Harbor.
14. **Nester, E.W.,** 1981. Manual of method for general bacteriology, American Society for Microbiology, Washington.
15. **Palleroni, N.J.,** 1978. The *Pseudomonas* group, p. 1-80. Meadfield Press, Durham.
16. **Smith, P.K.,** 1985. Measurement of protein using bicinchoninic acid, *Anal. Biochem.* **150**, 76-85.
17. **Stumpf, D.K. and R.H. Burris,** 1981. Organic acid content of soybean: Age and source of nitrogen. *Plant Physiol.* **68**, 989-991.
18. **Thomas, C.M.,** 1981. Molecular genetics of broad host range plasmid RK2. *Plasmid* **5**, 10-19.
19. **Williams, P.A. and K. Murray,** 1974. Metabolism of benzoate and the methylbenzoates by *Pseudomonas putida* (arvilla) mt-2: Evidence for the existence of a TOL plasmid. *J. Bacteriol.* **120**, 416-423.

(Received April 6, 1994)
(Accepted May 3, 1994)

초 록: *Pseudomonas fluorescens*에 있는 하나의 Plasmid가 말론산 대사에 관련된 유전자를 가지고 있다는 증거

김유삼* · 김은주 (연세대학교 이과대학 생화학과 및 생물산업소재 연구센터)

말론산을 탄소원으로 하여 성장하는 *Pseudomonas fluorescens*에서 60 kb 정도의 plasmid를 발견하였다. 이 plasmid를 curing한 *Pseudomonas*는 malonate 배지에서 자라지 못하였고 plasmid도 소실되었다. 또한 이 plasmid를 transformation과 conjugation으로 각각 *E. coli*와 cured *P. fluorescens*로 이동시킨 결과 이 plasmid를 받은 transformed *E. coli*와 conjugant *P. fluorescens*는 malonate를 탄소원으로 하여 성장하였고 malonate 대사에 관련된 효소인 malonate decarboxylase와 acetyl-CoA synthetase의 활성이 측정되었다. Western blotting을 통하여 tansformed *E. coli*에서 *P. fluorescens*와 동일한 acetyl-CoA synthetase가 malonate에 의해 induction됨을 확인하였다.