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Acquisition of the Ability to Grow Under Autotrophic Conditions by Heterotrophic Bacteria Through the Introduction of DNA Fragments from Hydrogen-Oxidizing Bacteria

Fusako Umeda, Kazuhiro Yano, Noritsugu Gohda, Kikuno Fujihara, Isamu Maeda, Kiyohito Yagi,* Yoshiharu Miura, and Tadashi Mizoguchi

Faculty of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565 Japan

ABSTRACT

Pseudomonas oxalaticus OX1 carrying recombinant plasmid pFUS and 29 gram-negative bacteria were used as donor and recipients, respectively, for conjugations. The plasmid pFUS contains genes necessary for chemolithoautotrophic growth in a hydrogen-oxidizing bacterium, *Alcaligenes hydrogenophilus*. By the conjugal transfer of plasmid pFUS, 12 strains acquired the ability to grow in a mineral salts medium under a gas mixture containing H_2, O_2, and CO_2. *Pseudomonas cepacia* carrying pFUS had hydrogenase and ribulose-1,5-bisphosphate carboxylase activities, and could grow at a rate almost equivalent to the growth rates of ordinary hydrogen-oxidizing bacteria. *Pseudomonas acidophila* carrying plasmid pFUS expressed indigenous antibiotic secretion under chemolithoautotrophic conditions. Plasmid pFUS could be stably maintained in these strains in the absence of selection pressure created by the addition of an antibiotic or chemolithoautotrophic conditions. This study shows that introduction of DNA fragments responsible for chemolithoautotrophic growth into a producer of valuable substances could be a promising technique for CO_2 recycling.

Index Entries: Hydrogen-oxidizing bacteria; *Alcaligenes hydrogenophilus*; in vivo cloning; chemolithoautotrophy; carbon dioxide.

INTRODUCTION

The most important greenhouse gas, carbon dioxide, is emitted when fossil fuels are burned. The rate at which carbon dioxide has accumulated in the atmosphere closely parallels the increased use of energy, and efforts to stabilize emis-

*Author to whom all correspondence and reprint requests should be addressed.

sions of carbon dioxide have now assumed great urgency. Recently developed technology enables carbon dioxide to be recovered from the gases emitted by steam power plants *(1)*. In light of this, we have been trying to develop means of recycling recovered carbon dioxide as useful materials, such as antibiotics and biodegradable polymer, using microorganisms. Hydrogen-oxidizing bacteria can grow in a mineral salts medium with carbon dioxide as a sole carbon source and hydrogen as an energy source. These characteristics could be used for carbon dioxide recycling to produce many valuable substances. *Alcaligenes hydrogenophilus* is a gram-negative, facultative, hydrogen-oxidizing bacterium *(2)* harboring a megaplasmid, pHG21-a, which has genes encoding carbon fixation (Cbb), hydrogen oxidation (Hox), and formate oxidation (Fox) *(3)*. Recently, we succeeded in cloning the DNA region of the plasmid responsible for carbon fixation and hydrogen oxidation in an IncP1 R-plasmid, R68.45, and the recombinant plasmid pFU7 was obtained *(4)*. Further, a σ^{54}-like chromosome gene that acted in the expression of *hox* was inserted into pFU7, and the recombinant plasmid pFUS was obtained *(5)*. Since the R-plasmid is self-transmissible and has a broad host range *(6)*, the recombinant plasmid can be transferred to a wide variety of bacteria by conjugation. In this study, we attempted to confer chemolithoautotrophic growth (Aut) ability to bacteria which can secrete antibiotics and to confirm their production from carbon dioxide.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

pFUS has three resistance markers derived from R68.45: resistance against ampicillin (Ap), kanamycin (Km), and tetracycline (Tc). pFUS was kept not in *A. hydrogenophilus*, but in *Pseudomonas oxalaticus* OX1 because the transfer gene of R68.45 was highly expressed in strain OX1 *(7)*. *P. oxalaticus* strain OX1-MH212 (an auxotrophic mutant of strain OX1) carrying pFUS was used as the donor. *Bacillus subtilis* IFO3513 was used as the test bacterium for antibiotics secreted from conjugants containing pFUS.

Conjugation

Conjugations were done by filter mating at 30°C. L-broth overnight cultures of donor and recipient were mated on a membrane filter in an L-plate overnight. Bacteria were then suspended in 4 mL of water, and the bacterial suspension was spread on selective medium plates at 0.1 mL/plate using an appropriate dilution. Transconjugants were selected by chemolithoautotrophic growth and antibiotic resistance markers. Chemolithoautotrophic growth was performed in a mineral salts medium under a gas mixture containing H_2, O_2, and CO_2 at a ratio of 8:1:1 (v:v:v) *(8)*.

Selection

Selection by antibiotic resistance markers was carried out under heterotrophic conditions. Glucose, fructose, or malate was used as the substrate, which was added at a final concentration of 0.2% (w/v). Antibiotics were used at the following final concentrations (μg/mL): Ap, 100; Km, 100; Tc, 10. The antibiotic and substrate used for each recipient strain are given in the Tables 1, 2, and 3. Transconjugants were cultivated at 37°C for *Escherichia coli* strains and 30°C for other strains.

Table 1
Strains Acquiring Chemolithoautotrophic Growth Ability
by Introduction of the Recombinant Plasmid pFUS

Strain	Autotrophic conditions, CFU/mL	Antibiotic resistance, CFU/mL[a]
P. acidophila IFO 13774	2.8×10^5	1.0×10^8 (G/Tc)
Pseudomonas aeruginosa IFO 13561	1.5×10^6	n.t.[b]
P. cepacia IFO 14595	4.0×10^4	1.8×10^7 (G/Km)
P. cepacia IFO 15124	2.0×10^4	n.t.
P. dacunhae IAM 1152	1.5×10^6	3.6×10^6 (M/Tc)
Pseudomonas fluorescens ATCC 39502	3.2×10^2	1.1×10^4 (M/Km)
P. mesoacidophila IFO 13884	1.3×10^3	n.t.
Pseudomonas oleovorans ATCC 13474	2.8×10^5	3.1×10^7 (M/Tc)
Pseudomonas putida TN100	2.5×10^3	2.7×10^8 (F/Km)
Pseudomonas stutzeri ATCC 31147	1.2×10^3	n.t.
Rhodopseudomonas rubrum NCIB8255	8.4×10^2	1.4×10^4 (M/Km)
Rhodopseudomonas spheroides NCIB8253	1.2×10^2	1.4×10^7 (M/Km)

[a]The carbon source for heterotrophic cultivation and the antibiotic used are indicated as follows: malate (M), glucose (G), fructose (F), kanamycin (Km), tetracyclin (Tc).
[b]n. t., not tested.

Table 2
Strains Acquiring Organotrophic Growth Ability
by Introduction of the Recombinant Plasmid pFUS

Strain	Formate oxidation, CFU/mL	Antibiotic resistance, CFU/mL[a]
P. aeruginosa IFO3898	4.5×10^6	2.6×10^6 (G/Km)
P. fluorescens NCIB8286	1.4×10^6	2.9×10^6 (F/Tc)
P. oleovorans IFO13583	3.7×10^4	0.2×10^5 (M/Km)
P. putida ATCC15175	1.0×10^6	1.0×10^9 (M/Km)
P. putida IFO3738	6.6×10^2	5.4×10^6 (G/Km)
Pseudomonas sp. TA8	6.4×10^5	2.2×10^7 (F/Km)

[a]Abbreviations are the same as in Table 1.

Inhibition of Bacterial Growth

The Aut$^+$ transconjugants obtained in this study were cultivated under chemolithoautotrophic conditions. Cells were eliminated from the culture broth by centrifugation. The culture filtrate was adjusted to pH 7.0 and concentrated by evaporation. The mineral salts medium was used as the standard. Cell suspension of *B. subtilis* was inoculated into L-plate, and cups were put on the plate. Samples were poured gently into the cups, and the plate was incubated at 30°C overnight.

Table 3
Strains Acquiring Antibiotic Resistance
by Introduction of the Recombinant Plasmid pFUS

Strain	Antibiotic resistance,[a] CFU/mL
E. coli IFO 3210	4.3×10^8 (G/Tc)
E. coli IFO 3552	8.3×10^8 (G/Tc)
E. coli IFO 13500	1.4×10^7 (G/Ap)
E. coli IFO 13502	1.7×10^7 (G/Ap)
Pseudomonas chlororaphis IFO 3521	2.2×10^8 (G/Km)
P. fluorescens ATCC 13475	1.4×10^7 (M/Km)
P. fluorescens IFO 3925	1.3×10^8 (G/Km)
P. fluorescens IFO 13658	5.5×10^7 (G/Km)
Pseudomonas mendocina IFO 14162	6.8×10^5 (G/Km)
Pseudomonas reptilivora IFO 3461	2.4×10^8 (G/Km)
Pseudomonas sp. IFO 13302	1.2×10^6 (M/Km)

[a]Ap, ampicillin; other abbreviations are the same as in Table 1.

Assays

Activities of hydrogenases and ribulose-1,5-bisphosphate carboxylase were measured as described previously *(4)*. Protein was measured by the method of Lowry et al. *(9)*.

RESULTS

Conjugations were done as described in Materials and Methods using *P. oxalaticus* strain OX1-MH212 carrying pFUS as the donor. Table 1 shows strains acquiring Aut ability by conjugal transfer of pFUS. In the first eight strains listed in Table 1, the transfer of pFUS was determined by Aut and the antibiotic resistance marker, whereas in the remaining four strains, the transfer was determined only by Aut, because they indigenously possessed resistance to Ap, Km, and Tc. Strains in Table 1 formed colonies on the selection plate consisting of mineral salts and antibiotic under a mixture H_2, O_2, and CO_2. Aut ability and antibiotic resistance were derived from inserted DNA in pFUS and the vector plasmid, R68.45, respectively. Comparing the Aut^+ and antibiotic-resistant transconjugants, *Pseudomonas dacunhae* IAM1152 exhibited almost the same number of transconjugants under both selections, but the other five strains had several orders of magnitude fewer transconjugants in the selection by Aut than that by the antibiotic-resistant marker. Although 29 strains in all acquired antibiotic resistance, only 12 could express chemolithoautotrophy. Since pFUS encodes formate-dependent organoautotrophic growth (Fox) ability as well as that of Hox, we next examined whether the remaining 17 strains carrying pFUS had organoautotrophic growth ability. Cells were inoculated on plates containing formate and antibiotic, and cultivated aerobically for 1 wk at 30°C. Table 2 shows that six strains carrying pFUS could grow on formate as both an energy and carbon source. The remaining 11 strains, as shown in Table 3, only expressed antibiotic resistance in the region of R.68.45.

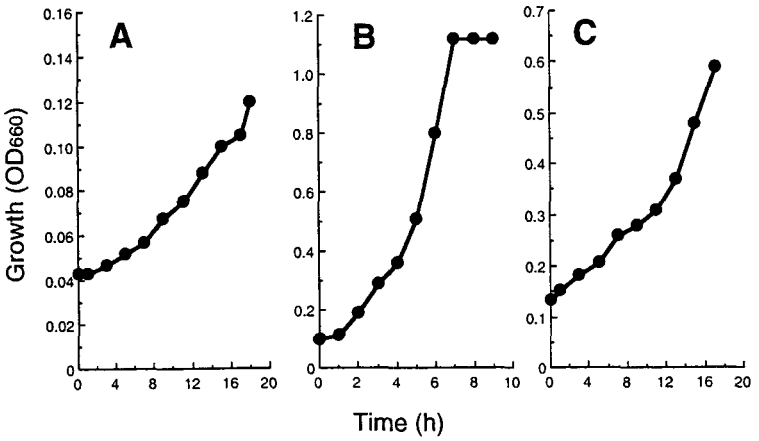

Fig. 1. Chemolithoautotrophic growth of *Pseudomonas* strains carrying pFUS. Recombinant *P. acidophila* **(A)**, *P. cepacia* **(B)**, and *P. mesoacidophila* **(C)**, were each inoculated into test tubes containing 6 mL of mineral salts medium and cultivated at 30°C under a mixture of H_2, O_2, and CO_2 at a ratio of 8:1:1 (v:v:v). The OD at 660 nm was measured at the times indicated.

Out of the 12 strains listed in Table 1, we selected 3, *Pseudomonas acidophila*, *Pseudomonas cepacia*, and *Pseudomonas mesoacidophila*, which produce the antibiotics G-6302, Altericidins, and SB-72310, respectively, for further experiments. The three strains were inoculated into liquid medium containing only mineral salts and cultivated at 30°C under an H_2, O_2, and CO_2 gas mixture. There was no growth of their parent strains under these conditions in the absence of pFUS (data not shown). As shown in Fig. 1, the Aut characteristics of the three strains were also confirmed in liquid culture. Table 4 shows the growth rates and activities of hydrogenases and ribulose-1,5-*bis*-phosphate carboxylase, which are key enzymes of H_2 oxidation and CO_2 fixation, respectively. The growth and activities varied among the three strains. Notably, *P. cepacia* carrying pFUS grew fastest at a rate almost equivalent to the growth rates of ordinary hydrogen-oxidizing bacteria, whereas *P. acidophila* containing pFUS grew very slowly and had only very slight enzyme activity for Aut ability. The stability of pFUS was examined in the three strains by checking the Aut ability after heterotrophic cultivation. The strains were cultivated overnight in L-broth and inoculated onto L-plates after appropriate dilution. One hundred colonies were picked, transferred onto a mineral-salts-medium plate, and cultivated for several days under a mixture of H_2, O_2, and CO_2. All tested colonies of three strains were confirmed to be grown under chemolithoautotrophic conditions (data not shown). Therefore, pFUS could be stably maintained in these strains in the absence of selection pressure, such as the addition of antibiotic or chemolithoautotrophic conditions.

The ability of the three strains containing pFUS to secrete antibiotics was examined in the culture filtrate obtained from chemolithoautotrophic culture as described in Materials and Methods. Contrary to the Aut ability, the culture filtrate from *P. acidophila* showed strong antibiotic activity toward the test bacterium, *B. subtilis* (Fig. 2). The filtrate from *P. cepacia* did not cause an inhibition zone (data not shown).

Table 4
Chemolithoautotrophic Growth and Activities of Hydrogenases and Ribulose-1,5-Bisphosphate Carboxylase in Recombinant Strains

Strain harboring pFUS	Doubling time, h	RuBPCase, U/g protein[a]	Hydrogenase, U/mg protein SH[b]	MH[c]
P. acidophila IFO13774	11.2	7.8	n.d.[d]	0.03
P. cepacia IFO14595	4.4	14.0	0.46	1.60
P. mesoacidophila IFO13884	8.6	19.0	0.36	1.41

Pseudomonas strains carrying pFUS were cultivated under chemolithoautotrophic conditions.
[a]Ribulose-1,5-bis-phosphate carboxylase.
[b]Soluble hydrogenase.
[c]Membrane-bound hydrogenase.
[d]Not detected.

DISCUSSION

Hydrogen-oxidizing bacteria belonging to diverse taxonomic groups have been reported, including both gram-positive and gram-negative bacteria. It is of interest to elucidate how many taxonomic groups containing hydrogen-oxidizing bacteria can express *aut* genes from *A. hydrogenophilus*. In this study, we chose gram-negative, aerobic, heterotrophic bacteria as the recipients for the following reasons: *A. hydrogenophilus* is gram-negative and the R68.45 used as cloning vector has a broad host range among gram-negative bacteria; the donor and recipient must grow on a nutrient broth like L-broth during conjugation under aerobic conditions, and the Aut^+ transconjugant of each recipient has to be cultivated under an atmosphere of 10% O_2. *Pseudomonas*, *Rhodopseudomonas*, and *Escherichia* strains were used as the recipients. *P. oxalaticus* OX1-MH212 carrying pFUS was used as the donor, and the appearance of Aut^+ transconjugants was determined. Conjugants were divided into three groups:

1. Those expressing Aut ability;
2. Those expressing only Fox ability; and
3. Those expressing only antibiotic resistance.

In Group 1, a comparison of the number of colonies selected by Aut and by antibiotic resistance shows that *aut* genes might be deleted from pFUS during conjugation. However, once pFUS was successfully transferred into the recipient, it was shown to be stably maintained even in heterotrophic conditions. Six strains belonging to Group 2 did not have Aut ability, but did possess Fox ability. Hydrogen-oxidizing bacteria can oxidize formate by formate dehydrogenase to form CO_2, and assimilate it via the Calvin cycle *(10)*. These strains did not grow under chemolithoautotrophic conditions, probably because *hox* genes in pFUS could not be expressed or deleted during the conjugation. Since there were 11 strains belonging to Group 3, an extensive analysis to determine the reason why Aut could not be expressed will have to be made.

It was confirmed that metabolically engineered *P. acidophila* and *P. mesoacidophila* secreted antibiotics during chemolithoautotrophic growth. There was an inverse relationship between the growth rate and the amount of antibiotic. Since rapidly growing cells cannot afford to secrete a secondary metabolite, a two-stage system in which growth and production are separated might be effective.

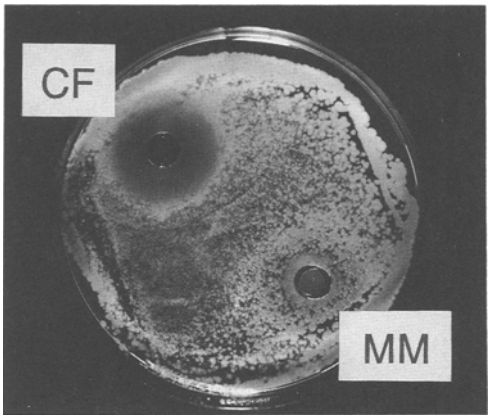

Fig. 2. Growth inhibition of *B. subtilis* IFO3513 by culture filtrate from *P. acidophila* IFO13774 carrying pFUS grown under chemolithoautotrophic conditions. The culture filtrate from *P. acidophila* IFO13774 carrying pFUS (CF) and mineral salts medium (MM) were used as the sample and control, respectively.

In this study, we demonstrated the possibility of constructing a novel system to recycle CO_2. Recently, many attempts have been made to produce valuable products from CO_2 using hydrogen-oxidizing bacteria *(11,12)*. The characteristic feature of this work is that a producer of valuable products can be changed to a chemolithoautotroph by the introduction of the DNA fragment responsible for Aut ability, thereby enabling many substances to be selected as targets for production.

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