TRANSFORMATION OF BACILLUS SUBTILIS INTO HYDROCARBON-RESISTANT BACILLUS

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INTRODUCTION

Studies of microbial degradation of pollutants have provided much critical information needed for ex situ waste treatment and for in situ bioremediation (Hinchee et al. 1994; CISB 1993). A marine bacteria, Acinetobacter calcoaceticus RAG-1 was identified as an oil-degradation microorganism in an earlier study (Reisfeld et al. 1972; Rosenberg et al. 1979a, b; Shabtai et al. 1985), and actively applied for bioremediation of the beaches in Alaska after the 1989 Exxon Valdez oil spill. Several other naturally occurring bacteria of the genus Pseudomonas were also discovered which utilize oil as their carbon energy source (Harvey et al. 1990). In this study, we report the discovery of another important soil bacteria, B. subtilis, which can be transformed with, or fused with the plasmid, pTV1Ts to obtain different capabilities to catabolize and degrade petroleum hydrocarbons.

MATERIALS AND METHODS

Bacterial strains and plasmids

A bacterial strain, PY313, containing plasmid, pTV1Ts ($Tn917Erm^{r}Cm^{r}Ts^{rep}$) (Youngman et al. 1983, 1989) was obtained from Dr. Youngman. The host cell for the plasmid were originated from BD170 (trpC2 thr5), one of B. subtilis 168 derivative (Dubnau et al. 1969). The recipients of the plasmid in the current transformation experiment were B. subtilis 168 prototrophic (1A2) from the Bacillus Genetic Stock Center (BGSC) and a strain of (MR1) wild type selenite-hyperresistant B. subtilis which was isolated from Mississippi River (Yang et al. 1994). The wild type selenite hyperresistant spore-forming bacteria was classified into the genus of Bacillus subtilis according to the difference in carbon source utilizations by microplate incubation method of Biolog Inc. (Miller et al. 1991; Klinger et al. 1992).

Culture media and reagents

Luria-Bertani broth (LB) containing 0.5% NaCl, 0.8% tryptone, 0.3% yeast extract was used for regular liquid culture. Tryptose Blood Agar Base (TBAB) medium containing 0.5% NaCl, 1.0% tryptose, 0.3% beef extract and 1.5% agar was used for regular agar plate culture. Selection media for antibiotics were made by inclusion of 2 ug/ml erythromycin and 12.5 ug/ml chloramphenicol in LB (LBEC) or in TBAB (TBABEC). For selection of selenite resistant cells, TBAB containing 10 mM sodium selenite was used (TBAB +10 mM Se). Hydrocarbon catabolism was tested by measuring the growth of bacteria in the TBAB (regular growth medium) or in Basic Medium (BM) containing 79.8 mM K_2HPO_4, 44.1 mM KH_2PO_4, 15.1 mM $(NH_4)_2SO_4$, 6.5 mM Sodium Citrate, 0.8 mM $MgSO_4$, 0.1 mM $Ca(NO_3)_2$, 0.1 mM $MnCl_2$, 0.001 mM $FeSO_4$, and 2% agar with the addition of 2% or 4% (v/v) petroleum hydrocarbons. Various petroleum hydrocarbons, such as Penzoid motor oil, Jandorf turbine oil, Amoco type II diesel oil, and Exxon unleaded regular gasoline were mixed into TBAB or BM with or without the addition of Tween-80.

Preparation of plasmids

For isolation of pTV1Ts, a single colony of PY313 was cultured in LB containing erythromycin (2 ug/ml) and chloramphenicol (12.5 ug/ml)[LB+EC] at 30°C with 250 rpm for 24 hours in a shaker incubator. After centrifugation at 3000 rpm for 20 minutes, bacterial pellet was obtained for preparation of plasmid according to a modified method using alkaline lysis of host bacteria (Lee et al. 1990). The purified plasmid DNA was precipitated and kept in 70% ethanol until the time of further experiment.

Transformation of B. subtilis prototrophic or wild type selenite-hyperresistant bacilli (MR1) with pTV1Ts

After overnight culture of a single colony of 168 prototrophic or MR1 in 12 ml LB at 30°C for 16 hours, a staionary phase of bacterial growth was obtained. After centrifugation at 3000 rpm for 20 minutes, the cell pellet was isolated. One ml of LB was used to resuspend the cell pellet, another ml was used to dissolve plasmid DNA following removal of 70% ethanol from the plasmid storage tube. Following the combination of cell suspension with the plasmid preparation, shaking culture was continued for another 6 to 12 hours in a smaller tube (13 x 100 mm) for cellular uptake of plasmid DNA with or without the help of electroporation. Thereafter, 200 ul of the mixture was spread cultured on MG plate containing erythromycin (2 ug/ml) and chloramphenicol (12.5 ug/ml) for expression of transformed cells at 30° C (Yang et al. 1996). A derivative of B. subtilis 168 prototrophic transformed by pTV1Ts was designated as WH-0, and another derivative of wild type bacillus MR-1 transformed by the same plasmid was named WH-1, respectively.

Mutagenetic treatment of transformed cell

A colony of WH-0 or WH-1 on TBABEC agar was isolated into the LBEC broth for shaking culture (250 rpm) at 30°C for 24 hours to stationary phase. After centrifugation at 3,000 rpm for 20 minutes, cell pellet was resuspended into fresh LBEC Kept at 48°C. Thereafter, shaking culture was continued at 48°C for another 24 hours for growth of mutants with insertion of plasmid (Youngman et al. 1983, 1989). After centrifugation at 3,000 rpm for 20 minutes, mutants were isolated in the cell pellet. Following resuspension of the cell pellet in 5 ml of LB containing 15% glycerol, the cell suspension was divided into many microtubes (0.5 ml/tube) for storage at -80°C. For selection of mutants with strong capability of degrading hydrocarbons, the frozen storage of mutants in a microtube was rapidly thawed and diluted 50 to 200 times in LB medium and spread cultured on TBAB plate containing 10 mM selenite to select for the selenite resistance cell. The selenite resistant mutants were then patch-cultured with toothpick at corresponding graphic locations on selection media containing TBAB with or without antibiotics to select for antibiotic-resistance mutants. Thereafter, mutants were tested for growth in TBAB or BM containing various petroleum hydrocarbons.

Assessment and Monitoring of Bacterial Growth on Agar Plate

A single colony of the bacterium was isolated and patched on the center of each plate in a set of 4 to 6 plates containing TBAB or BM with the addition of various amounts of hydrocarbons or selenite for assessment of their effect on growth or metabolism. Colony size was daily monitored by photography of the plate for record of the colony image. The colony size was thereafter calculated by the number of grids(1.25 x1.25 mm) filled by images in a transparent section paper.

RESULTS

B. subtilis 168 prototrophic was transformed into WH-0 with antibiotic resistance characters inherited from the plasmid. Unexpectedly, the transformed cell was greatly enhanced for its growth capability in the presence of hydrocarbons such as diesel oil or motor oil also (Table 1). MR-1 was also transformed into WH-1 with similar resistance to both erythromycin and chloramphenicol. In addition, growth enhancement was also found by inclusion of diesel oil in the medium (Table 2). A proportional increase of bacterial growth in correlation with the concentration of diesel oil from 2% to 4% n TBAB was seen in the culture (Tables 1 and 2). In spite of growth enhancement by the inclusion of diesel oil in the media, both strains of transformed cells, WH-0 and WH-1 were unable to grow in the basic medium without carbon sources (BM) or in such medium with the inclusion of diesel oil. Nevertheless, when WH-1 was subjected to mutagenetic treatment and selected for selenite-hyperresistant ability, a few of them (20%) were capable of growing in the BM with the addition of either 2% motor oil, diesel oil, or gasoline oil as a sole source of carbon for energy and carbon metabolism (Table 3). The most difficult petroleum hydrocarbon for catabolism appeared to be gasoline oil. Some of selenite-hyperresistant mutants were still unable to catabolize petroleum hydrocarbons as a sole source of carbon metabolism (Table 4). In spite of the successful mutagenetic experiment in WH-1, none of the mutagenetic experiment in WH-0 were able to construct any strains capable of catabolizing gasoline oil as a sole source of carbon metabolism.

DISCUSSION

Both transformation and mutagenetic experiments suggested the possible existence of hydrocarbon-catabolizing gene(s) in the plasmid, pTV1Ts. Whether

these gene functions are the additional function of erythromycin resistance gene (ribosyl demethylase) or other unidentified gene in the Tn 917 remained to be solved.

Several enzymatic functions related with the catabolism of fatty acid are closely related with the catabolism of hydrocarbons because of their close similarity in the chemical structures. Beta-oxidation is well known for their catabolic process for the formation of a high energy thioester bond to be used for aerobic production of ATP in the glyoxylate cycle. Defect in alpha-oxidation of fatty acid (such as phytanic acid) and alcohol (such as phytol) is also known for a inherited metabolic disease (Refsum's disease) related with neurologic malfaunction (Stanbury et al. 1978). A minor pathway for the oxidation of fatty acids by oxidation of epsilon terminal carbon was also observed in microsomes fraction of the rat liver (McGarry et al. 1980). Gluconeogenesis from acetyl coA via anabolic pathway of the glucose fermentation is considered to be responsible for synthesis of glucose and other important amino acids from the fatty acids or alcohols in the reversed direction of regular metabolism. It is theoretically possible for petroleum hydrocarbons to be substituted for fatty acids and other nutritional components (carbohydrates and proteins) as an alternate carbon source for entire cell metabolism in some extreme metabolic conditions. This may take place if enzymes were available to convert the terminal carbon into methanol or carboxyl group by epsilon oxidation. The possible existence of the ribosomal methyl-transferase gene (erm) located in Tn917 (Perkins et al. 1986) of plasmid pTV1Ts may also be responsible for this process. It is also possible that hydrogen peroxide radicals produced by extraordinary active biological process may contribute to the oxidation of the terminal methyl group of hydrocarbon by the Harber-Weiss reaction mediated by intracellular Fe^{2+} and H_2O_2 (Mello-Filho et al. 1984). Indeed, hydrogen peroxide created in the process of oxidative phosphorylation has been known to oxidize a variety of substrates including phenol, formic acid, formaldehyde, and alcohol. In microbodies isolated from fungal zoospores, catalase as well as malate synthetase and isocitrate lysase were used as mediators for oxidizing process (Powell 1976).

SUMMARY AND CONCLUSION

Successful transformation of B. subtilis into hydrocarbon resistant bacilli were achieved by introduction of a plasmid, pTV1Ts. This study indicates that plasmid, pTV1Ts is capable of transforming B. subtilis into potential petroleum hydrocarbon-catabolizing microorganisms. Furthermore, this study demonstrates that petrochemical hydrocarbon can be used as the sole carbon source for energy metabolism in a derivative of B. subtilis following transformation and adequate mutagenetic treatment. Genes that are involved in petroleum hydrocarbon degradation or oxidation may have existed on a still-unidentified portion of the plasmid or related with the erythromycin-resistant gene (methyl-transferase function). If the plasmid has been inserted into adequate location(s) of the host cell chromosome, the host cell may obtain an unique capability of catabolizing petroleum hydrocarbons as a single carbon source for energy metabolism.

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REFERENCES

Committee on In Situ Bioremediation (CISB). 1993. In situ bioremediation: when does it work? Water Science and Technology Board, Commission on Engineering and Technical System, National Research Council. Washington, D. C.: National Academy Press.

Dubnau, D., R. Davidoff-Abelson, and I. Smith. 1969. Transformation and transduction in bacillus subtilis: evidence for separate modes of recombinant formation. J. Mol.Biol. 45: 155.

Harvey, S., I. Elashvili, J. J. Valdes, D. Kamely, and A. M. Chakrabarty. 1990. Enhanced removal of Exxon Valdez spilled oil from Alaskan Gravel by a microbial surfactant. 8: 228-230.

Hinchee, R. E., D. B. Anderson, F. B. Metting, Jr., and G. D. Sayles, eds. 1994. Applied biotechnology for site remediation. Boca Raton, Florida: Lewis Publishers.

Klinger, J. M., R. P. Stowe, D. C. Obenhuber, T. O. Groves, S. K. Nishra, and D. L. Pierson. 1992. Evaluation of the biolog automated microbial identification system. Applied and Environmental Microbiology. 58: 2089-2092.

Lee, S-y, and S. Rasheed. 1990. A simple procedure for maximum yield of high-quality plasmid DNA. Biotechnique 9: 676-679.

McGarry, J. D. and D. W. Foster. 1980. Regulation of hepatic fatty acid oxidation and ketone body production. Ann. Rev. Biochem. 49: 395.

Mello-Filho, A.C., and R. Meneghini, 1984. In vitro formation of single -strand breaks in DNA by hydrogen peroxide is mediated by the Haber-Weiss Reaction. Biochemica et Biophysica Acta. 781: 56-63.

Miller, J. M. and D. L. Rhoden. 1991. Preliminary evaluation of Biolog, a carbon source utilization methods for bacterial identification. J. of Clinical Microbiology. 29: 1143-1147.

Perkins, J. B. and P. Youngman. 1986. Construction and properties of Tn917-lac, a transposon derivative that mediates transcriptional gene fusion in Bacillus subtilis. Proc. Natl. Acad. Sci. USA 83: 140-144.

Powell, M. J. 1976. Protoplasma. 89: 1.

Reisfeld, A., E. Rosenberg, and D. Gutnick. 1972. Microbial degradation of crude oil: factors affecting the dispersion in sea water by mixed and pure cultures. App. Microbiol. 24: 363-368.

Rosenberg, E., A. Zuckerberg, C. Rubinovitz, and D. L. Gutnick. 1979a. Emulsifier of Arthrobacter RAG-1: isolation and emulsifying properties. Appl. Environ. Microbiol. 37: 402-408.

Rosenberg, E., A. Perry, D. T. Gibson, and D. L. Gutnick. 1979b. Emulsifier of Arthrobacter RAG-1: specificity of hydrocarbon substrate. Appl. Environ. Microbiol. 37: 409-413.

Shabtai, Y. and D. L. Gutnick. 1985. Exocellular esterase and emulsan release from the cell surface of Acinetobacter calcoaceticus. J. Bacteriol. 161: 1176-1181.

Stanbury, J. B., J. B. Wyngaarden and D. S. Fredrickson. 1978. The metabolic basis of inherited diseases. New York: McGraw-Hill.

Yang, W. H., A. Baaree, J. R. Yang, and A. Yee. 1994. High selenium concentration and selenite-hyperresistant bacteria in the lower stream of Mississippi River. In Proceedings of the Mississippi Water Resources Conference 24: 37.

Yang, W. H., A. Baaree, L. Drumond, and J. R. Yang. 1996. Transformation of b. subtilis.(In preparation for publication).

Youngman, P., J. V. Perkins, and R. Losich. 1983. Genetic transposition and insertional mutagenesis in bacillus subtilis with Streptococcus faetalis transposon Tn917. PNAS 80: 2305.

Youngman, P., H. Poth, B. Green, K. York, G. Olmedo, and K. Smith. 1989. Method for genetic manipulation, cloning, and functional analysis of sporulation gene in bacillus subtilis, In Regulation of procaryotic development. Smith, I., R. A. Slepecky, and P.Setlow, eds. p. 65. Amer. Soc. for Microbiology. Washington, D.C.

Table 1. Growth characteristics of B. subtilis 168 prototrophic and WH-0(a derivative of 168 pro. transformed by pTV1Ts) at 30° C.

Bacteria	Media	Colony Size (mm^2) During Culture		
		Day 1	Day 2	Day 3
168 pro	TBABEC[1]	0.	0.	0.
	TBAB	7.8	20.3	54.6
	+10 mMSe[2]	4.7	7.8	12.5
	+2% DO[3]	12.5	56.2	343.2
	+4% DO[4]	331.0	371.0	975.0
	2% DOBM[5]	0.	0.	0.
WH-0(1)	TBABEC	6.2	6.2	29.6
	TBAB	10.9	28.1	56.2
	+10 mMSe	4.7	15.6	15.6
	+2% DO	214.1	428.1	554.7
	+4% DO	935.9	1793.8	2182.8
	2% DOBM	0.	0.	0.
WH-0(2)	TBABEC	6.2	10.9	17.2
	TBAB	18.8	29.6	34.3
	+10 mMSe	4.7	12.5	18.8
	+2% DO	18.8	371.0	722.0
	+4% DO	946.8	1987.5	2587.5
	2% DOBM	0.	0.	0.

Note 1. TBAB agar with the inclusion of 2 ug/ml erythromycin and 12.5 ug/ml chloramphenicol.
Note 2. TBAB agar with the inclusion of 10 mM sodium selenite.
Note 3. TBAB agar with the inclusion of 2% (v/v) diesel oil.
Note 4. TBAB agar with the inclusion of 4% (v/v) diesel oil.
Note 5. Basic medium with the inclusion of 2% (v/v) diesel oil.

Table 2. Growth characteristics of MR-1 and WH-1 (a derivative of MR-1 transformed by pTV1Ts) at 30°C.

Bacteria	Media	Colony Size (mm^2) During Culture		
		Day 1	Day 2	Day 3
Incubation at 30°C				
MR-1	TBABEC[1]	0.	0.	0.
	TBAB	93.0	450.0	721.9
	+10 mMSe[2]	35.9	114.1	243.8.
	+2% DO[3]	58.3	178.1	438.0
	+4% DO[4]	104.7	320.3	555.0
	2% DOBM[5]	0.	0.	0.
WH-1	TBABEC	15.2	58.4	118.0
	TBAB	55.6	232.5	590.4
	+2% DO	115.6	429.6	1418.0
	2% DOBM	0.	0.	0.

Note 1. TBAB agar with the inclusion of 2 ug/ml erythromycin and 12.5 ug/ml chloramphenicol.
Note 2. TBAB agar with the inclusion of 10 mM sodium selenite.
Note 3. TBAB agar with the inclusion of 2% (v/v) diesel oil.
Note 4. TBAB agar with the inclusion of 4% (v/v) diesel oil.
Note 5. Basic medium with the inclusion of 2% (v/v) diesel oil.

Table 3. Growth characteristics of WH-1-M5 mutant which can use various petroleum hydrocarbons as a single carbon source for catabolism (incubated at 38° C).

Media Containing	Colony Size (mm^2) During Culture							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
TBAB only	29.7	51.6	54.7	120.3	146.9	184.4	226.6	285.9
+EC[1]	6.3	18.8	23.4	43.8	64.1	65.6	65.6	95.3
+0.1 mM Se[2]	12.5	25.0	21.9	43.8	65.6	79.4	84.4	121.5
+1.0 mM Se[3]	9.4	17.2	17.2	37.5	50.0	65.6	70.3	78.1
+10 mM Se[4]	4.7	7.8	7.8	17.2	29.7	34.4	45.3	60.9
+2% DO[5]	15.6	35.9	76.6	134.4	164.1	240.6	328.1	390.6
BM only	0.	0.	0.	0.	0.	0.	0.	0.
+2% DO[6]	4.7	9.4	18.8	35.9	39.1	42.2	53.9	59.4
+2% MO[7]	4.7	4.7	7.8	11.7	20.3	20.3	34.4	34.4
+1% GO[8]	6.3	6.3	6.3	12.5	18.8	18.8	23.4	23.4
+2% GO[9]	10.5	12.5	18.8	20.3	39.1	39.1	39.1	39.1

Note 1. TBAB agar with the inclusion of 2 ug/ml erythromycin and 12.5 ug/ml chloramphenicol.
Note 2. TBAB agar with the inclusion of 0.1 mM sodium selenite.
Note 3. TBAB agar with the inclusion of 1 mM sodium selenite.
Note 4. TBAB agar with the inclusion of 10 mM sodium selenite.
Note 5. TBAB agar with the inclusion of 2% diesel oil.
Note 6. Basic medium with the inclusion of 2% diesel oil.
Note 7. Basic medium with the inclusion of 2% motor oil.
Note 8. Basic medium with the inclusion of 1% gasoline oil.
Note 9. Basic medium with the inclusion of 2% gasoline oil.

Table 4. Growth characteristics of WH-1-M12 mutant which cannot use any petroleum hydrocarbons as a single carbon source for catabolism (incubated at 38° C).

Media Containing	Colony Size (mm^2) During Culture							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
TBAB only	54.7	234.4	644.5	737.5	1953.1	3144.5	4101.6	4707.0
+EC[1]	14.1	64.1	125.0	234.4	206.3	507.8	664.1	918.0
+0.1 mM Se[2]	48.4	156.3	351.6	605.5	820.3	1230.5	1777.3	2148.4
+1.0 mM Se[3]	39.1	103.1	151.6	312.5	312.5	449.2	585.9	800.8
+10 mM Se[4]	17.2	65.6	125.0	214.8	253.9	351.6	449.2	546.9
+2% DO[5]	57.8	371.1	703.0	977.5	1387.4	2128.9	2226.6	2281.3
BM only	0.	0.	0.	0.	0.	0.	0.	0.
+2% DO[6]	0.	0.	0.	0.	0.	0.	0.	0.
+2% MO[7]	0.	0.	0.	0.	0.	0.	0.	0.
+1% GO[8]	0.	0.	0.	0.	0.	0.	0.	0.
+2% GO[9]	0.	0.	0.	0.	0.	0.	0.	0.

Note 1. TBAB agar with the inclusion of 2 ug/ml erythromycin and 12.5 ug/ml chloramphenicol.
Note 2. TBAB agar with the inclusion of 0.1 mM sodium selenite.
Note 3. TBAB agar with the inclusion of 1 mM sodium selenite.
Note 4. TBAB agar with the inclusion of 10 mM sodium selenite.
Note 5. TBAB agar with the inclusion of 2% diesel oil.
Note 6. Basic medium with the inclusion of 2% diesel oil.
Note 7. Basic medium with the inclusion of 2% motor oil.
Note 8. Basic medium with the inclusion of 1% gasoline oil.
Note 9. Basic medium with the inclusion of 2% gasoline oil.