CONSTRUCTION OF BENZENE-CATABOLIZING BACILLI BY TRANSFORMATION AND GENE-FUSION WITH PLASMIDS

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INTRODUCTION

Aerobic degradation of aromatic hydrocarbons by bacteria was first demonstrated in the early 1900s. Thereafter, various strains of bacteria and fungi capable for degradation of aromatic hydrocarbons were isolated (Gibson et al. 1984). Nevertheless, bacteria capable for degradation of long aliphatic hydrocarbons such as petroleum oil were discovered only recently. A marine bacteria, Acinetobacter calcoaceticus RAG-1, was identified as an oil-degradation microorganism in earlier studies (Reisfeld et al. 1972; Rosenberg et al. 1979 a, 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 which utilize oil as their carbon energy source (Harvey et al. 1990), were also discovered. In the previous studies (Yang et al. 1996a, b) we indicated that it was able to convert the bacilli into gasoline-catabolizing bacilli by plasmid-mediated transformation and gene-fusion with plasmid pTV1Ts. Further study indicated that following such treatment, the bacilli obtained their capability to catabolizing benzene as the only carbon source in the basic medium. It was also possible to enhance the benzene-catabolizing capability of those pTV1Ts-fused bacilli by retransformation and refusion with the additional new plasmid, pLTV1 which contain large portion of similar DNA molecules with pTV1Ts.

MATERIALS AND METHODS

Bacterial strains and plasmids

A bacterial strain, PY313, containing plasmid, pTV1Ts ($Tn917Erm^rCm^rTs^{rep}$) (Youngman et al. 1983, 1989) was obtained from Dr. Youngman. The host cells for the plasmid were originated from BD170 (trpC2 thr5), one of Bacillus subtilis 168 derivatives (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 bacterium was classified into Bacillus mycoides 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% trypton, 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. Hydrocarbon catabolism was tested by measuring the growth of bacteria in the 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% (v/v) benzene or 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 incubation of a single colony of 168 prototrophic or MR1 (B. mycoides) in 12 ml LB at 30°C for 16 hours, a staionary phase of bacterial growth was obtained. The cell pellet was isolated after centrifugation at 3000 rpm for 20 minutes. One ml of LB was used to resuspend the cell pellet; another ml was used to dissolve plasmid DNA immediately after the removal of 70% ethanol from the plasmid 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 the 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 (B. mycoides) transformed by the same plasmid was named WH-1, respectively.

Isolation of Mutants with Gene Fusion

For isolation of mutants in this study, either WH-0 or WH-1 were cultured in the LB+EC medium for 24 hours at the temperature at or below 30°C and at 250 rpm for increase of the number of transformed cells as well as plasmid-fused cells in the initial step. After centrifugation at 3000 rpm for 20 minutes, a cell pellet was resuspended into the newly prepared LB+EC medium for shaking culture at 48°C for another 24 hours. The replication of plasmid was inhibited by the elevation of culture temperature which activated the Ts^{rep} gene and subsequently resulted in the death of transformed cells. Consequently, only mutants with plasmid-fusion to bacterial chromosome selectively grew in this medium (Youngman et al. 1983, 1989). At the end of culture, the increased mutants were collected by centrifugation and resuspended in 5 ml of LB containing 15% glycerol for frozen storage at -80°C. After thawing these cells, they were spread cultured on TBAB plate containing 10 mM selenite to select for selenite resistance bacterial colony. Thereafter, the selenite-resistant mutants were selected for antibiotic resistances. Bacteria were further tested for growth in TBAB or BM containing various petroleum hydrocarbons by patch-culture technique.

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 benzene or petroleum hydrocarbons or selenite for assessment of their effect on growth or metabolism. Immediately after the patch culture of the bacterial colony for test, silicon rubber was used to seal the space between side walls of top and bottom plastic petri dish to prevent evaporation of volatile hydrocarbons. 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. The transformed cells were greatly enhanced for its growth capability in the presence of aromatic hydrocarbon (benzene) as well as aliphatic hydrocarbons (diesel oil or motor oil) (Table 1). Another strain of selenite hyperresistant bacillus, MR-1 (B. mycoides), was transformed into WH-1 and was able to grow in the presence of antibiotics (erythromycin, chloramphenicol) as well as diesel oil or motor oil. But the WH-1 was incapable for growth in the presence of benzene as well as gasoline (Table 2). Assessment of bacterial growth by daily measurement of colony size demonstrated that there are proportional increase of bacterial growth in correlation with the concentration of diesel oil at 2% to 4% level (Tables 1 and 2). In spite of growth enhancement by the inclusion of diesel oil in the enriched media such as tryptose blood agar base (TBAB), both strains of transformed cells, WH-0 and WH-1 were unable to grow in the basic medium (BM) with the inclusion of benzene, diesel oil, gasoline oil. Nevertheless, when WH-1 was subjected to mutagenetic treatment with gene-fusion and selected for selenite-hyperresistant ability, a few of them (20%) were capable of growing in the BM with the addition of either 1% or 2% benzene as well as 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 benzene or gasoline oil. Many of the selenite-hyperresistant mutants were still unable to catabolize benzene or petroleum hydrocarbons as a sole source of carbon in basal medium (Table 4). In spite of the successful construction of benzene-catabolizing bacilli from WH-1, none of WH-0 were able to convert into benzene-catabolizing bacilli following similar treatment with plasmid fusion. It suggests the importance of gene background in the host cells and of the adequate location of gene fusion to be responsible for the mutant to obtain capability of hydrocarbon catabolism (Table 2). The origin of transformed cell, WH-1, is the wild type bacillus, B. mycoides, with high selenite-resistance, and strong growth capability, whereas the origin of transformed cell, WH-0, is low in selenite-resistance, and not strong in growth capability.

DISCUSSION

Both transformation and mutagenetic experiments suggested that the possible existence of benzene-catabolizing gene(s) in the plasmid, pTV1Ts. Whether these gene functions are the additional function of erythromycin resistance gene (ribosyl demethylase), oxygenases, or other unidentified gene in the Tn 917 remained to be solved. For aerobic degradation of aromatic hydrocarbon by bacteria, numerous investigators have examined metabolic pathway of benzene in various microorganisms. Those studies indicated that there are many

different pathways which can be used by bacteria in the degradation of aromatic hydrocarbons. Benzene is degraded by conversion first into either catechol or protocatechulate before oxidative ortho- or meta- ring cleavage with molecular oxygen serving as both a terminal electron acceptor and a reactant (Baker and Herson 1994). A group of oxygenases is responsible for these reaction in benzene degradation. It is still unknown whether a similar group of oxidases will be responsible for the oxidation of aliphatic hydrocarbons resulting in the catabolism of gasoline, diesel oil, or motor oil, as well as benzene in the aromatic hydrocarbon.The possible existence of alkanase or the ribosomal methyl-transferase gene (erm) located in Tn917 (Perkins et al. 1986) of plasmid pTV1Ts may also conduct this process. A minor pathway for the oxidation of fatty acids by oxidation of epsilon terminal carbon (McGarry et al. 1980) may also be responsible for oxidative cleavage of benzene. The bacilli transformed and fused with the plasmids have shown petroleum oil-degradation capability (Yang et al. 1996) as well as benzene-degradation capability in the current experiment. The acetyl-CoA and succinate produced as a result of ring cleavage can be further oxidized via the Krebs cycle and electron transfer chain system. 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 therefore possible for benzene to substitute other fatty acids, carbohydrates, or 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. It may also be possible that peroxide radicals produced by extraordinary active biological process may contribute to the oxidation of the aromatic 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 phenols, formic acid, formaldehyde, and alcohol. In the microbodies isolated from the fungal zoospores, catalase, malate synthetase, and isocitrate lysase were used as the enzyme mediating of the oxidation process (Powell 1976).

SUMMARY AND CONCLUSION

B. subtilis 168 prototrophic was transformed into WH-0 with increased resistance to benzene. However, the transformed cells were still incapable to grow in the BM medium with either benzene or with any other petroleum hydrocarbon as the only source of carbon for metabolism (Table 1). Similar procedure in a wild type selenite hyperresistant bacilli (B. Mycoides, MR-1) was capable to increase resistance to benzene also. However, these transformation procedures were not able to convert the bacilli into benzene-catabolizing bacilli (Table 2). Further step in the gene-fusion did not produce benzene-catabolizing bacilli from WH-0 cells, but did produce some (nearly 20%) of benzene catabolizing bacilli from WH-1 (Table 3). Most of pTV1Ts-fused WH-1 is not benzene-catabolizing bacilli as indicated in Table 4. But some of pTV1TS-fused WH-1 cells could catabolize benzene or other petroleum hydrocarbons in the BM medium (STSF#4, STSF#5). Re-transformation and re-fusion of the pTV1Ts-fused bacilli with other plasmid, $pLTV_1$ could enhance the benzen-catabolizing capability of either STSF#4 or STSF#5 to higher levels (DTDF#1, DTDF#2, Table 3).

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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	6.8	22.2	56.4
	+10 mMSe[2]	4.7	7.8	12.5
	+1% BZ[3]	12.4	48.8	123.6
	+2% BZ[4]	75.0	132.0	260.0
	2% BZBM[5]	0.	0.	0.
WH-0(1)	TBABEC	5.8	10.2	18.6
	TBAB	11.4	26.4	58.4
	+10 mMSe	4.2	11.2	14.4
	+1% BZ	118.6	224.4	318.8
	+2% BZ	257.4	496.4	894.4
	2% BZBM	0.	0.	0.
WH-0(2)	TBABEC	5.8	11.2	19.6
	TBAB	17.2	26.4	35.2
	+10 mMSe	4.4	11.8	19.6
	+1% BZ	108.4	239.0	382.4
	+2% BZ	280.8	526.5	946.2
	2% BZBM	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 1% (v/v) benzene.
Note 4. TBAB agar with the inclusion of 1% (v/v) benzene.
Note 5. Basic medium with the inclusion of 2% (v/v) benzene

Table 2. Growth characteristics of MR-1 and WH-1 (a derivative of MR-1 transformed by pTV1Ts) in TBAB or BM containing diesel oil or benzene 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
	+2% BZ[4]	42.4	120.6	288.6
	2% DOBM[5]	0.	0.	0.
	2% BZBM[6]	0.	0.	0.
WH-1	TBABEC	15.2	58.4	118.0
	TBAB	55.6	232.5	590.4
	+2% DO[3]	115.6	429.6	1418.0
	+2% BZ[4]	98.4	389.2	1189.4
	2% DOBM[5]	0.	0.	0.
	2% BZBM[6]	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.
Note 6. Basic medium with the inclusion of 2% (v/v) diesel oil.

Table 3. Comparison of growth of pTV1Ts-fused mutants (STSF#4, STSF#5)and pTV1Ts- and pLTV1-double fused mutants (DTDF#1, #2) in BM containing 2% benzene, or in TBAB containing other contents(STSF#4 mutant only).

Media Containing	Colony Size (mm^2) at Various Days of Cultures							
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
TBAB only	14.1	40.6	70.3	121.9	156.5	215.6	223.4	259.4
+EC[1]	9.4	18.8	39.1	57.8	68.9	87.5	90.6	112.5
+10 mM Se[2]	3.1	4.7	4.7	6.3	6.3	7.8	7.8	12.5
+2% DO[3]	18.8	50.0	104.4	164.1	234.4	326.6	436.6	546.9
BM only	0.	0.	0.	0.	0.	0.	0.	0.
+2% DO[4]	4.7	9.4	20.3	37.5	39.1	50.0	50.0	59.4
+2% GO[5]	20.3	31.3	39.1	39.1	39.1	39.1	40.6	40.6
+2% BZ[6]		44.2	50.1	60.1	68.8	78.5	110.7	118.0
+2% BZ[7]		19.6	30.7	37.1	51.5	68.6	68.7	78.6
+2% BZ[8]	1.6	6.7	37.4	117.2	150.4	195.4	234.6	248.4
+2% BZ[9]	1.6	1.6	38.6	179.4	281.6	624.6	706.5	718.4

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% diesel oil.
Note 4. Basal medium agar with the inclusion of 2% diesel oil.
Note 5. Basal medium agar with the inclusion of 2% gasoline oil for growth of STSF#4 mutant.
Note 6. Basal medium agar with the inclusion of 2% benzene for growth of STSF#4 mutant.
Note 7. Basal medium agar with the inclusion of 2% benzene for growth of STSF#5 mutant.
Note 8. Basal medium agar with the inclusion of 2% benzene for growth of DTDF#1 mutant.
Note 9. Basal medium agar with the inclusion of 2% benzene for growth of DTDF#2 mutant.

Table 4. Growth characters of STSF#12 mutant which cannot use either aromatic hydrocarbon (benzene) or petroleum hydrocarbons as a single carbon source for catabolism.

Media Containing	Colony Size (mm^2) at Various Days of Cultures							
	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
+10 mM Se[2]	17.2	65.6	125.0	214.8	253.9	351.6	449.2	546.9
+2% DO[3]	57.8	371.1	703.0	977.5	1387.4	2128.9	2226.6	2318.4
+2% BZ[4]	44.6	128.4	372.6	589.4	789.8	1126.4	1534.6	1845.2
BM only	0	0	0	0	0	0	0	0
+2% DO[5]	0	0	0	0	0	0	0	0
+2% GO[6]	0	0	0	0	0	0	0	0
+2% BZ[7]	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 10 mM sodium selenite.
Note 3. TBAB agar with the inclusion of 2% diesel oil.
Note 4. TBAB agar with the inclusion of 2% benzene.
Note 5. Basal medium agar with the inclusion of 2% diesel oil.
Note 6. Basal medium agar with the inclusion of 2% gasoline oil.
Note 7. Basal medium agar with the inclusion of 2% benzene.