CONSTRUCTION AND INDUCTION OF HYBRID BACILLI FOR CATABOLIZING BOTH NITROAROMATIC AND POLYAROMATIC HYDROCARBONS

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INTRODUCTION

Aromatic hydrocarbons particularly insoluble polyaromatic hydrocarbons (PAHs) have been accumulated in nature mainly as a part of petroleum as the results of metabolic activities of living organisms on this planet. Serious contamination of environment with those compounds has been resulted from the explosive use of petroleum by human activity. The extensive application of those PAHs in industry for wood preservation and road construction seriously contaminated the environment . Rosenfeld and Plumb (1991) reported that PAHs were present in over 20% of soil components in the wood treating site tested. PAHs contamination of soils and ground water are of particular concern because many of those compounds are known as carcinogens. Most of nitroaromatic compounds are artificially produced in large quantity by industry for plastics, explosives, pharmaceuticals, and pesticides (Bryant and DeLuca, 1991; Hallas and Alexander, 1983; Kedderis et al., 1988; Nishino and Spain, 1993). As a result, large amount of nitrobenzene is contaminating the environment at a rate of tens of millions of pounds annually (U.S. Environmental Protection Agency, 1978). In addition, nitrated polycyclic aromatic hydrocarbons are also formed during a variety of combustion processes causing serious environmental pollution (Rafii, et al., 1991). Aerobic degradation of aromatic hydrocarbons by a microorganism was first demonstrated in the early 1900s. Since then, various strains of bacteria and fungi capable of catabolizing aromatic hydrocarbons were isolated (Gibson et al. 1984). Biodegradation of aliphatic hydrocarbons (gasoline and diesel oil) by bacteria were also developed contributing greatly in bioremediation of enviroment of disaster by oil spill (Reisfeld et al. 1972; Rosenberg et al. 1979 a, b; Britton 1984; Shabtai and Gutnick, 1985; Yang et al. 1996a,b). The application of gene-recombination to obtain hybrid bacteria for aromatic hydrocarbon catabolism was first demonstrated in Pseudomonas (Duque et al. 1993). Our earlier report indicated that both petroleum hydrocarbons (Yang et al. 1996a, b), and benzene (Yang et al. 1997) can be catabolized by hybrid of B. mycoides following transformation and gene-fusion of bacilli with either pTV_1Ts or $pLTV_1$.

The object of the current study is to investigate whether biodegradation of both nitroaromatic and polyaromatic hydrocarbons can be achieved in bacilli following gene-recombination with plasmids either by transformation or gene-fusion. Another object of current study is to examine which catabolic pathways are more favaorable for biodegradation of nitroaromatics or polyaromatics.

MATERIALS AND METHODS

Bacterial strains and plasmids

A bacterial strain, PY313, containing plasmid, pTV_1Ts ($Tn917Erm^r\ Cm^r\ Ts^{rep}$; 12.4 Kbp in DNA size) (Youngman et al. 1983, 1989) and a bacterial strain PY1177, containing plasmid, $pLTV_1$ ($Tn917Erm^rCm^rAmp^rTc^r\ LacZ^+\ Ts^{rep}$ 20.6 Kbp in DNA size)(Camilli et al. 1990) were obtained from Dr. Youngman. The host cells for the both plasmids were originated from BD170 (trpC2 thr5), one of B. 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 wild type selenite-resistant bacillus (MR1) which was isolated from Mississippi River. The selenite-resistant bacillus, MR-1 was classified as Bacillus mycoides (Yang et al. 1994) according to the difference in carbon source utilization by microplate incubation method of Biolog Inc. (Miller et al. 1991; Klinger et al. 1992).

Culture media, selection media and reagents

Luria-Bertani broth (LB) containing 0.5% NaC1, 0.8% tryptone, 0.3% yeast extract was used for

regular liquid culture. Tryptose Blood Agar Base (TBAB) medium containing 0.5% NaC1, 1.0% tryptose, 0.3% beef extract and 1.5% agar was used for regular agar plate culture. Selection media for bacteria resistant to antibiotics erythromycin and chloramphenicol were made by inclusion of 2 μg/m erythromycin and 12.5 μg/ ml chloramphenicol in LB (as LBEC) or in TBAB (as TBABEC). Chemically defined basic media for aerobic metabolism using either glucose (BM1α) or hydrocarbons (and BM1β) for the only carbon or energy sources in aerobic catabolism was previous investigation (Yang et al. 1997) and listed in Table 1. Chemically definded basic media for deniftrifying metabolism were designed for using nitrate as the nitrogen source, and hydrocarbon as carbon source in BM2β agar. In the BM3β agar, the nitrated hydrocarbon for testing is both the nitrogen and carbon source in that medium. The compositions of each basic media were listed in the Table 1. The agar used for making Basic Media was measured to the amount for forming 2.0% weight/volume concentration in the media. Following repeated washing with tap water for 6 times in 3 days, washing with distilled water were continued for 4 times. Afler sterilization in autoclave to the pressure of 20 lbs per square inch for 40 minutes of 2.5 % agar, all other mineral compositions glusose, citric acid and hydrocarbons were filtered through sterile filter apparatus into separate bottles. Afler he sterilized agar cooled down to the temperature below 70° C , other filter-sterilized components of medium will be added separately to prevent the formation of precipitate. The final concentrations of chemicals in BM2β agar are: 79.8 mM for K_2HPO_4 44.1 mM for KH_2PO, 32 mM for $NaNO_3$, 6.8mM for sodium citrate. 0.8 mM for $MgSO_4$, 0.1 mM for $Ca(NO_3)_2$ 0.1 mM for $MnCl_2$, 0.001 mM in $FeSO_4$, 0.32 mM for Na_2MoO_4 and 2% for agar. BM3β agar was prepared from all chemicals listed for preparation of BM2β agar except $NaNO_3$ and Na_2MoO_4. For preparation of regular basic medium, BM1α agar, in addition to all chemicals and agar in BM3β agar, 151.4 mM of NH_4SO_4 and 2% dextrose were included. In BM1α dextrose was used as the only carbon source for metabolism. In special basic media, BM2β or BM3β, either aliphatic or aromatic hydrocarbons or their derivatives were added to be used as the carbon source depending on the nature of experiment (Table 1). Selection media for bacteria resistant to antibiotics, erythromycin and tetracyclin were made by inclusion of 2μg/m erythromycin and 12.5 μg/m tetracyclin in LB (as LBET) or in TBAB (as TBABET).

Preparation of plasmids

For isolation of TV_1Ts, a single colony of PY313 was cultured in LBEC containing erythromycin (2 μg/ml" and chloramphenicol (12.5 μg/ml) at 30°C with 250 rpm for 24 hours. Afler 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. For isolation of plasmid, $pLTV_1$, a single colony of PY1177 was cultured in LBET containing erythromycin (2 μg/ml) and tetracyclin (12.5 μg/ml) at 30°C with 250 rpm for 24 hours. Afler centrifugation at 3000 rpm for 20 minutes, bacterial pellet was obtained for preparation of plasmid, $pLTV_1$ according to a modified method using alkaline lysis of host bacteria (Lee et al. 1990). The purified plasmid $pLTV_1$ was precipitated and kept in 70% ethanol until the time of further experiment.

Transformation of B. subtilis prototrophic or wild type selenite-hvnerresistant bacilli (MR1) with pTV_1Ts or $pLTV_1$

Afler overnight culture of a single colony of 168 prototrophic or MR1 in 12 ml LB at 30° C for 16 hours, a stationary phase of bacterial growth was obtained. Afler ccntrifugation at 3000 rpm for 20 minutes, a cell pellet was isolated. One ml of LB was used to resuspend the cell pellet, another ml was used to dissolve plasmid , pTV_1 Ts following

removal of 70% ethanol from the plasmid storage tube. Afler mixing cell suspension with the plasmid, pTV_1Ts suspension, the mixture was electroporated with 400 DCV for 100 msec. Shaking culture of the electroporated mixture was continued for another 6 to 12 hours in a smaller tube (13 x 100 mm) . Thereafter, 200 μl of the mixture was spread-cultured on BM1αEC agar plate containing erythromycin (2 μg/ml" and chloramphenicol (12.5 μg/ml" for formation of colonies on the agar plate at 28" C (Yang et al. 1996). For preparation of transformed bacilli for test, a single colony was isolated into 42.5 ml of LBEC broth for shaking culture at 29°C for 24 hours. Following the addition of 7.5 ml glycerol, the cell preparation in LBEC containing 15% glycerol was divided into tubes, 5 ml per tube for storage at -80°C until next experiment. For transformation of bacilli with plasmid $pLTV_1$, a colony of 168 prototrophic or MR1 was cultured in 12 ml LB broth at 29°C for 16 hours to stationary phase. After centrifugation at 3000 rpm for 20 minutes the supernatant was removed. One ml of the supernatant was used to resuspend the pellet, another ml of the supernatant was used to resuspend the plasmid, $pLTV_1$. Afler mixing cell suspension with the plasmid ($pLTV_1$) suspension, the mixture was electroporated with 400 DCV for 100 msec. Shaking culture of the treated mixture was continued for another 6 hours in a smaller tube (13 x 100 mm) . Thereafter, 200 μ of the mixture was spread-cultured on BM1αETagar plate (Table 1) containing erythromycin (2 μg/ml) and tetracyclin (12.5 μg/ml) for formation of colonies on the agar plate at 28° C (Yang et al. 1996). For preparation of transformed bacilli for test, a single colony was isolated into 42.5 ml of LBET broth for shaking culture at 29°C for 24 hours. Following the addition of 7.5 ml glycerol, the cell preparation in LBET containing 15% glycerol was divided into tubes 5 ml per tube for storage at -80°C until next experiment. A derivative of B. subtilis 168 prototrophic transformed by pTV_1 Ts was designated as WH-0, and another strain of wild type bacillus MR-1 (B. mycoides) transformed by the same plasmid, pTV_1 Ts was designated as WH-1 respectively. Similarly, a strain of B. subtilis 168 prototrophic transformed by $pLTV_1$ was designated as WH-2, and another strain of wild type bacillus MR-1 (B. mycoides) transformed by the same plasmid, pTV_1 Ts was designated as WH-3 respectively.

Testing of hydrocarbon catabolism or biodegradation by the application of test bacterial spots followed by application of hydrocarbon spots 24 hours later on chemically definded media

All test bacilli were cultured for 24 hours in shaker incubator at 200 rpm and 28°C to late staionary phase in LBEC or LBET containing antibiotics. Following the addition of 15% glycerol, the test bacilli were frozen in a deep freezer at -80°C until the time of use.

For the most common test, generally used template for 50 locations of bacterial inoculation positions were attached to the test agar plate. A test bacteria from the thawed frozen stock was applied to evenly located 9 LB spots (50μl per spot) respectively at the graphic positions designated as open circles in Figure 1 for incubation at designated temperature for 24 hours in various different basic media. Hydrocarbon dissolved or suspended as 50% concentration in dimethyl sulfoxide (DMSO) was applied 24 hours later to 4 spots (50 μ per spot) at the graphic locations designated as closed circles in Figure 1 on the test agar plates. After sealing of the space between the top and the bottom plate of the petri dish with silicone caulk, incubation of the agar plates was continued for 12 weeks with the observation and recording of the images on LB spots at the interval of 1or 2 weeks. General effect of bacterial spots to the hydrocarbons diffused to come from chemical spots from various directions and locations can be evaluated by this test method. The response of the growing bacterial lawn to the approaching hydrocarbon by diffusion could be recognized primary by color change caused by biodegradation of TNT molecule or by benzene with nitrate in the media during incubation. Secondary effect of the

aromatic hydrocarbon catabolism to bacteria could be observed as the formation of bacterial colonies with color changes on the colonies at various metabolic conditions during 12 weeks of incubation. In some occasions, test bacteria were applied spirally at various distances from the center of the plate. Following a day of culture, test hydrocarbon was applied as the only spot to the center of the plate (Figure 2), so that the spatial effect of applied hydrocarbon to the bacterial spots could be better studied.

RESULTS

It has been demonstrated in separate experiment in pretests that bacterial colonies capable of catabolizing toxic TNT can be only cloned by sequential applications of bacterial spots and chemical spots with 24 hours or longer delay. In additionm, some adequate spatial distance between bacterial spots and chemical spots. The very toxic effect of the TNT to the bacilli can be modified by such procedures to induce the necessary bacterial enzymes to catabolize that toxic molecule. Based on this sequential application methods, the following experiments were performed to examine the necessary gene backgrounds of bacilli and the necessary catabolic conditions for catabolizing nitroaromatic and polyaromatic hydrocarbons.

Native bacilli of either B. subtilis (168 prototrophic) or B. mycoides (MR-1) were incapable of catabolizing aromatic hydrocarbon (Benzene) in the chemically definded medium, BM1β which was designed for aerobic metabolism (Group 1, Tables 2, 3, 4, and 5). Both bacilli were incapable of catabolizing aromatic hydrocarbon in the medium, BM2β which was designed for denitrification metabolism (Group 2, Tables 2,3, 4, and 5) also. Neither colony formation nor color formation were observed following incubation of such native bacilli during 12 weeks of incubation at 28°C. After transformation of those bacilli with either pTV_1Ts or $pLTV_1$, transformed bacilli were not capable of catabolizing benzene as the only carbon source in the aerobic metabolism using BMIp as the medium (Group 4, Tables, 1, 2, 3, and 4). Elevation of incubation temperature to 38°C for transformed bacilli in B. mycoides (MR-1) to have gene-fusion with either plasmid pTV_1Ts or $pLTV_1$, however resulted in the formation of some hybrids capable of catabolizing benzene in BMIp with the colony formation rates of 0.94 per million (Group 12, Table 3) and 0.85 per billion (Group 12, Table 5) respectively. Gene fusion of B. subtilis with either plasmid pTV_1Ts or $pLTV_1$, by elevation of temperature to 38°C did not results in the formation of hybrid bacilli capable of catabolizing benzene in BMIp (Group 12, Table 2, Table 4). Nevertheless, gene-fused hybrids of B. mycoides were capable of catabolizing benzene in denitrification medium, BM2β more effectively with higher colony formation rates (CFR of 1.39/billion for Group13, Table 5; 1.54/billion for Group 13, Table 5) than similar hybrids can do in the aerobic catabolism in BMIp (CFR 0.94/billior for Group 12, Table 2; 0.85/billior for Group 12, Table 4). In addition, the plasmid-fused hybrids of B. subtilis were also capable of catabolizing benzene in BM2β at the incubation temperature of 38°C with the comparable colony formation rates (1.94/million for Group13, Table 2; 2.56/million for Group 13, Table 4). Denitrification catabolism was not only preferable for plasmid-fused hybrids, but also suitable for the plasmid-transformed bacilli to catabolize benzene (Group 6 in Tables 2, 3, 4, and 5) and to catabolize toluene also (Group 7 in Tables 2, 3, 4, and 5). Denitrification catabolism was also very effective for catabolizing insoluble PAH,such as benzopyrene even at incubation temperature of 28°C which was very close to the regular environment temperature. The colony formed at the initial stage of bacterial growth were all white in color suggesting a complete biodegradation of the hydrocarbons without formation of colorful nitro compounds inside the colony. Nevertheless, with the incubation period prolonged to 12 weeks, gradual accumulation colorful metabolites increased. There is a formation of deep brown color for colonies formed by catabolism of benzene or toluene in the BM2β but not in BM1β. There is the formation of yellow brown color for colonies formed by catabolism of benzopyrene in BM2β.

A spectacular changes in the color of bacterial spots and bacterial colonies, however, were observed during the experiment for catabolizing TNT by the transformed bacilli (Group 5, Tables

2, 3, 4, and 5) or plasmid-fused bacilli (Group 10, Tables **2,** 3, 4 , and 5) in the denitrification medium, BM2β or BM3β. In those media for denitrification of TNT, brown color spots were formed initially by the lawn of growing bacteria on the LB spots for biodegradation of TNT diffusing from the neighboring chemical spots. Color change on the LB spots was followed by colony formation with characteristic color formation also, Colorless, white colonies were gradually formed on the background of brown color initially formed by a lawn of bacteria growing for biodegradation of TNT on the LB spots at the early stage of incubation. After 8 weeks of incubation, the retardation in the growth of bacterial colonies resulted in the change to red brown. The color change and colony formation accompanied with growth of transformed bacilli in BM2β (Group 9, Table **2,** 3, 4, and 5) or in BM3β (Group 11, Table **2,** 3, 4, and 5) could be effectively blocked by the addition of equivalent amount of nitrite to nitrate in the denitrification media, BM2β or BM3β. The addition of equivalent amount of nitrite to nitrate on BM2β also effectively inhibit the color change and the colony formation induced by the catabolism of TNT at higher temperature at 38°C in BM2β medium alone (Group 16, Table **2,** 3, 4, and 5).

DISCUSSION

Although hybrids of B. mycoides were capable of catabolizing hydrocarbons in such aerobic environment using BM1β **as** medium for incubation, colony formation rates were lower and bacterial growths were poorer in such medium.

The difficulty of oxygen in air to diffuse through benzene-containing medium in BM1β for catabolizing benzene or other aromatic or aliphatic hydrocarbons may be the disadvantage of using the aerobic mechanism for biodegradation of benzene. By contrast, the easiness to obtain large amount of soluble oxygen released from the nitrate compound in the medium (BM2β) to apply for benzene calibration in denitrification catabolism may be the advantage for taking such mechanism in aromatic hydrocarbon catabolism. The qualified bacilli for such catabolism should possessed both gene capability of reducing nitrate (by reductase in denitrification enzymes) and another gene capability of catabolizing benzene molecule (by oxidase including dioxygenase for formation of catechol to split the ring structure). Apparently, plasmid pTV_1Ts or $pLTV_1$ contains the necessary genes for both enzymes near to transposon fragment of the plasmid. Transformation of bacilli with either plasmid will enable the transformed bacilli to perform such catabolism in BM2β.

Such enzyme capabilities for denitrification are particularly important for TNT-catabolism. None of hybrid bacilli were capable of catabolizing TNT in the aerobic condition using BM1p (Group 3, Tables **2,** 3, **4,** and 5). In fact, reduction and release of some nitro groups from the TNT molecule are frequently monitored in the biodegradation of TNT by bacteria (McCormick et al. 1976). The release of nitro group from the TNT appeared to be absolutely necessary precondition before the modified molecule will be reactive to the second enzyme for catabolism of aromatic ring structure by dioxygenase for oxidizing to catechol and further splitting of the catechol into aliphatic hydrocarbons to be catabolized in citric acid cycle.

Earlier study by Duque et al (1993) demonstrated that Pseudomonas sp. clone A can grow on medium using TNT as nitrogen source and fructose as carbon sourcce. By transformation of the Pseudomonas clone A with TOL plasmid. pWWO-Km, the hybrid were capable of mineralizing TNT **as** the only carbon source **and** nitrogen source.

Degradatiopn of two- or three-ring aromatic compounds has been shown to be a popular phenomena among aerobic bacteria. As in the case with aerobic degradation of monocyclic aromatic compounds, the primary basic reaction is the enzymatic effect of dioxygenase form catechol in one of the aromatic ring. Further oxidation of the catechol will result in the fission of the ring structure and the metabolic intermediates will be feed into the Krebs cycle (Gibson and Subramanian, 1984). Biodegradation of PAHs under strictly anaerobic conditions has not been reported. Mihelicic and Luthy (1988) demonstrated that degradation of naphthalene could occur under denitrifying conditions, although no degradation of naphthalene or anthracene was found under strictly anaerobic (sulfate-reducing or methanogenic) conditions. In contrast, naphthol

was degraded under both denitrifying and strictly anaerobic conditions. Benzopyrene degradation by soil bacteria (Litchfield et al,1992, Miller et al., 1988; Werner et al., 1991) and by Estruarine microorganisms (Shiaris, 1989)were also reported. Study on the biotransformation of nitrated aromatics such as 2,4,6-trinitrotoluene (TNT) and nitrobenzene by bacteria demonstrated either aerobic or anaerobic metabolic pathway for degradation (Crawford, 1995; Marvin-Sikkema t al., 1994; Michaels and Gottschalk, 1995; Preuss and Rieger, 1993; Spain, 1995; Stahl and Aust, 1995, Pasti-Grigsby et al., 1996). Reduction and release of nitro groups as nitrite was reported in aerobic microbial degradation of some nitroaromatics (McCormick et al. 1976). Reduction of the nitrogroup is a common first step in the biotransformation of nitroaromatic compounds, either leading to mineralization of the compounds (Groenwegen et al., 1992; Nishino and Spain, 1993) or leading to accumulation of dead-end products. Many of them are cytotoxic or mutagenic (Bryant C. and McFlory. 1991; McCoy et al., 1990; Narai, N., S. Kitamura, and K. Tasumi. 1984).

In the current experiment, TNT has been shown to be colorful biological marker for monitoring both dinitrification effect and catabolizing effect of microorganism to the compounds. By demonstrating brown color over the growing bacterial lawn over LB spots , bacteria are responding to the diffused TNT with enzyme for reduction or removal of nitro group for modification of TNT molecule. By demonstrating white colony formation on the background of brown, bacteria are catabolizing effectively for mineralization of TNT. Effectiveness of catabolism can be evaluated by how many colonies will be formed by unit number of bacteria to response, and to what degree the brown color of degrading TNT to be degraded to colorless or light yellow on the LB spots.

CONCLUSION

Hybrid bacilli capable of catabolizing both nitroaromatic and polyaromatic hydrocarbons have been successfully constructed by transformation of either B. subtilis or B. mycoides with either plasmid. pTV_1Ts or plasmid, $pLTV_1$. Following such gene-recombination, hydrocarbon-catabolizing mechanism in the hybrid bacilli could be induced efficiently by sequential application of bacterial spots to BM2β agar plate first for starvation in denitrification environmet followed by application of hydrocarbons spots 24 hours later for induction. Without transformation, both strain of bacilli were incapable of catabolizing TNT in the chemically defined media either with denitrification (BM2β) or with aerobic catabolism (BM1β). Both native strains of bacilli were also incapable of catabolizing aromatic hydrocarbon such as benzene in the aerobic catabolism. Transformation alone was not enough to construct bacilli capable of catabolizing benzene in the aerobic catabolism (BM1β) . With the additional gene fusion, hybrid bacilli capable of catabolizing benzene in BM1β for aerobic catabolism were obtained in B. mycoides but not in the B. subtilis. Nevertheless, transformation of either bacilli with either plasmid were enough to construct bacilli capable of catabolizing TNT as well as benzene, toluene, and benzopyrene, in BM2β with denitrification catabolism. Gene-fusion of either bacilli with either plasmid were similarly effective to catabolize hydrocarbons, polycyclic hydrocarbons, and TNT as well as benzene in BM2β also. It appears that either plasmid pTV_1Ts or plasmid, $pLTV_1$. contains genes for denitrification and aromatic hydrocarbon catabolism near to the transposon sites which acted in favor of both hydrocarbon catablism and dentrification.

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REFERENCES

Bryant, C., and M. DeLuca. 1991. Purification and characterization of and oxygen-insensitive NAD(P)H nitroreductase from Enterobacter cloacae. J. Biol. Chem. 266: 4119-4125.

Bryant, C., and W. D. McElroy. 1991. Nitroreductases. P. 291-304. In F. Muller (ed.),

Chemistry and Biochemistry of flavoenzymes. Vol. II. CRC Press. Boca Baton, Fla.

Britton, L. N. 1984. Microbial Degradation of Aliphatic Hydrocarbons, in *Microbial Degradation of Organic Compounds,* D. T. Gibson (ed.). Marcel Dekker, Inc., New York pp. 89C129.

Crawford, R.L. 1995. Biodegradation of nitrated munition compounds and herbicides by obligatory bacteria. P. 87-98. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenu Press. New York

Duque, E., A. Haidour, F. Godoy, and J. L. Ramos. 1993. Construction of Pseudomonas hybid strain that mineralize 2,4,6-trinitrotoluene J. Bacteriology 175: 2278-2283.

Fernando, T., J.A. Bumpus, and S.D. Aust. 1990. Biodegradation of TNT (2,4,4-trinitrotoluene) by Phanerochaete chrysosporium. Appl. Environ. Microbiol. 56: 1666-1671.

Gibson, D. T. and V., Subramanian. 1984. Microbial Degradation of Asromatic Hydrocarbons, in Microbial Degradation of Organic Compoundsk D. T. Gibson, (ed.)/ Marcel; Dekker, Inc. New York, pp./ 182-252.

Groenewegen, P. E., J. P. Breeuwer, J. M. L. M. van Helvoot, A. A. M. Langenhoff, F. F. de Vries, and J. A. M. de Bont. 1992. Novel degradative pathway of 4-nitrobenzoate in Comamons acidovorans NBA-10. J. Gen, Microbiol. 138: 1599-1605.

Hallas, L. E., and M. Alexander. 1983. Microbial transformation of nitroaromatic compounds in sewage effluent. Appl. Environ. Microbiol. 45: 1234-1241.

Kedderis, G. L., L. S. Argenbright, and G. T. Miwa. 1988. Mechasnism of reductive activation of a 5-nitroimidazole in by flavoproteins: model studies with dithionite. Arch. Biochem. Biophys. 262: 40-48.

Lehrbach, P. R., J. Zeyer, W. Reineke, H.-J. Knackmuss, and K. N. Timmis. 1984. Enzyme recruitment in vitro: use of cloned genes to extend the range of haloaromatics degraded by Pseudomonas sp. strain B13. J. Bacteriol. 158: 1025-1032.

Lewis, T.A., Goszczynski, S., R. L. Crawford, R.A. Korus, and W. Admassu. 1996. Products of anaerobic 2,4,6-trinitrotoluene(TNT) transformation by Clostridium bifermentans. Appl. Environ. Microbiol. 62: 4669-4674.

Litchfield, C. D., D. S. Herson, K. H. Baker, R. Krishnamoorthy, and W. J. Zeli. 1992. Biotreatability Study and Field Pilot Design for a Wood Treating Site. Presented at IGT Symposium on Gas, Oil and Environmentl Biotechnology, September 21-23, 1992, Chicago, Ill.

Marvin-Sikkema, F.D., and J. A. M. de Bont. 1994. Degradation of nitroaromatic compounds by microorganisms. Appl. Microbiol. Biotechnol. 42:499-507.

McCormick, N. G., F. E. Feeherry, and H. S. Levinson. 1976. Microbial transformation of 2,4,6-trinitrotoluene and other nitroaromatic compounds. Appl. Environ. Microbiol. 31: 949-958.

McCoy, E. C., H. S. Rosenkranz, and P. C. Howard. 1990. Salmonella typhimurium TA100Tn5-1012, a strain deficient in arythydroxylamine O-esterificase exhibit a reduced nitroreductase activity. Mutat. Res. 243: 141-144.

Michels, J., and G. Gottschalk 1995. Pathway of of 2,4,6,-trinitrotoluene(TNT) degradation by by Phanerochaete chrysosorium , p. 135-149. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Press. New York.

Miller, R. M., G. M. Singer, J. D. Rosen, and R. Bartha, 1988. Photolysis primes biodegradation of benzo[a}pyrene. Appl. Environ. Microbiol. 54: 1724-1730.

Mihelicic, J. R. and R. C. Luthy. 1988. Degradation of polycyclic aromatic hydrocarbon compounds under various redox conditions in soil-water system Appl. Environ. Microbiol. 54: 1182-1187.

McGany, 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. Menegluni. 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. Preliminaiy evaluation of Biolog, a carbon source utilization methods for bacterial identification. J. of Clinical Microbiology. 29:1143-1147.

Nay, K. W., C. W. Randall, Jr., and P. W. King. 1974. Biological treatability of trinitrotoluene manufacturing waste-water. J. Water Pollut. Control Fed. 46: 485-497.

Nishino, S.F., and J. C. Spain. 1993. Degradation of nitrobenzene by Pseudomonas pseudoalcaligenes. App. Environ. Microbiol. 59: 2520-2525.

Narai, N., S. Kitamura, and K. Tasumi. 1984. A comparative study on 1-nitropyrene and nitrofurazone reductases on Escherichia coli. J. Pharm. Dyn. 7: 407-413.

Osmod, J. L., and R. E. Klausmeier. 1972. The microbial degradation of explosives. Dev. Ind. Microbiol. 14: 247-252.

Pasti-Grigsby. M.B., T. A. Lewis, D. L. Crawfoprd, and R. L. Crawford. 1996. Transformation of 2,4,6-trinitrotoluene (TNT) by actinomycetes isolated from TNT-contaminated and uncontaminated environments. Appl. Environ. Microbiol. 62: 1120-1123.

Perkins, 1. B., and P. Youngman 1986. Construction and properties of Tn9 I 7-lac, a transposon derivative that mediates transcriptional gene fusion in Bacillus subtilis. Proc. Nail. Acad. Sc. USA 83:140 144

Preuss, A., and P. G. Rieger. 1995. Anaerobic transformation of trinitrotoluene and other nitroaromatic compounds. P. 69-85. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Press. New York.

Rafii, F., W. Franklin, R. H. Heflich and C. E. Cerniglia. 1991. Reduction of nitroaromatic compounds by anaerobic bacteria isolated from the human gastrointestinal tract. Appl. Environ. Microbiol. 57: 962-968.

Ramos, J. L., A. Stolz, W. Reineke, and K. N. Timmis. 1986. Altered effector specificities in regulators of gene expression: TOL plasmid xy/S mutants and their use to engineer expansion of the range of aromatics degraded by bacteria. Proc. Natl. Acad. Sci. USA 83: 8467-8471.

Reinecke, W., and H. J. Knackmuss, 1979. Construction of haloaromatic-utilizing bacteria. Nature (London) 277: 385-386

Rosenfeld, J. K., and R. H. Plumb, Jr. 1991. Groundwater contamination at wood treatment facilities. Ground Water Monit. Rev. Winter: 133-140.

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. Rubinovit.z, and D.L. Gutnick. 1979a. Emulsifier of Arthrobacter RAG1:isolation and emulsifing properties. App. Environ. Microbiol. 37:402-408.

Rosenberg, E., A. Perzy, D. T. Gibson, and D. L. Gutnick. 1 979b. Emulsifier of Arthrobacter RAG-i: specificity of hydrocarbon substrate. Appl. Environ. Microbiol. 37:409-413.

Shabtai, Y. and D. L. Gutnick. 1985. Exocellular esters and emulsan release from the cell surface Acinetobacter calcoaceticus. 1. Bacteriol. 161:117~1181.

Shiaris, M. P. 1989. Seaqsonal biotransformation of naphthalene, phernanthrene and benzopyrewne in surfiucial estuarine sediments. Appl. Environ. Microbiol. 55: 1391-1399

Spain, J. C. 1995. Bacterial degradation of nitroaromatic compounds under aerobic conditions. P. 19-35. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Press. New York.

Stahl, J.C. and S. Aust 1995. Biodegradation of 2,4,6-trinitrotoluene by the white rot fungus Phanerochaete chrysosporium, p. 117-133. In J. C. Spain (ed.), Biodegradation of nitroaromatic compounds. Plenum Press. New York.

Traxler, R. W., E. Wood, and J. M. Delaney. 1974. Bacterial degradation of -TNT. Dev. Ind. Microbiol. 16: 71-76.

U. S. Environmental Protection Agency. 1978. Nitrobenzene Initial report of the TSCA interagency testing committee to the administrator. EPA 560-578/001. US. Environmental Protection Agency. Washington, D.C.

Werner, P. 1991. German Experiences in the Biodegradation of Creosoter and Gaswork-Specific Substances , in In Situ Bioreclamation: Applications and Investigations for Hydrocarbon and Contaminated Site Remediation, R. E, Hinchee and R. F. Olfenbuttel (eds.). Butterworth=Heinemann, Boston, pp. 496-516.

Yang, W. H., A. Baaree, J. R. Yang, and A. Yee. 1994. High selenium concentration and selenit hyperresistant bacteria in the lower stream of Mississippi River. In Proceedinas of the 24th

Mississippi Water Resources Conference 24:37-45
Yang, W. H., and J. R. Yang. 1996a. Transformation of Bacillus Subtilis into hydrocarbon-resistant bacillus In Proceedings of the 26th Mississippi WateResources Conference 26: 250-257.
Yang, W. H., and J. R. Yang. 1996b. Transformation of selenite-hyperresistant Bacillus subtilis into hydrocarbon-resistant bacteria for bioremediation a oil-contaminated site. In Proceedings of the 12th International Conference on Advanced Science and Technoloav. 221-236.
Youngman, P., J. V. Perkins, and R Losich. 1983. Genetic transposition and insertional mutagenesis in Bacillus subtilis with Streptococcus faetalis transposon Tn9 17. PNAS. 80: 2305.
Youngman, P., H. Poth, B. Green, K. York, G. Olmedo, and K. Smlth. 1989. Method for genetic manipulation, cloning, and functional analysis of Sporulation Gene in Bacillus subtilis, In ARegulation of Procarvotic Development, eds. Smith, I., R. A. Slepecky, and P. Setiow@. Washington, D. C. :Amer. Soc. for Microbiology. 65.

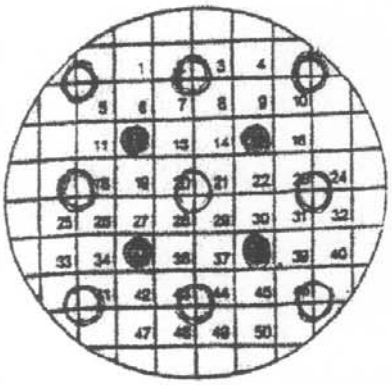

Figure 1. Template for application of 9 bacterial spots (open circles) and 4 chemical spots (closed circles).

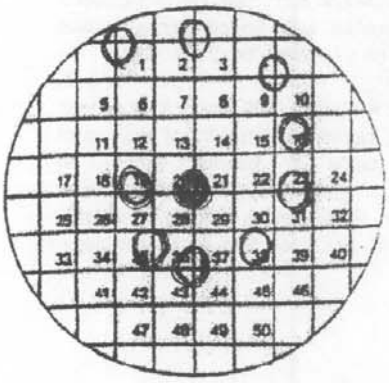

Figure 2. Template for application of 8 bacterial spots (open circles) and 1 chemical spot (closed circle).

Table 1. Chemically definded media to be included in agar plates for testing of hydrocarbon catabolism in bacilli.

Chem. Compositions	BM1α medium	BM1β medium	BM2β medium	BM3β medium
K_2HPO_4	79.8 mM	79.8 mM	79.8 mM	79.8 mM
NaH_2PO_4	44.1 mM	44.1 mM	44.1 mM	44.1 mM
Sodium citrate	6.8 mM	6.8 mM	6.8 mM	6.8 mM
$MgSO_46H_2O$	0.8 mM	0.8 mM	6.8 mM	6.8 mM
$(NH_4)_2SO_4$	15.1 mM	15.1 mM	15.1 mM	15.1 mM
$Ca(NO_3)_2$	0.1 mM	0.1 mM	0.1 mM	0.1 mM
$FeSO_4$	0.001 mM	0.001 mM	0.001 mM	0.001 mM
$MnCl_2$	0.1 mM	0.1 mM	0.1 mM	0.1 mM
Na_2MoO_4	----	----	0.32 mM	0.32 mM
$NaNO_3$	----	----	15.1 mM	----
Carbon source	Glucose	Hydrocarbons	TNT	TNT
Agar	2%	2%	2%	2%

Table 2. Construction of hydrocarbon catabolizing gene-mechanism on B. subtilis (168 protrophic) by transformation or gene-fusion of the bacilli with plasmid pTV1Ts. Bacilli were applied to 9 spots on the chemically definded media followed by application of hydrocarbons to 4 evenly-distributed on chemical spots for induction of hydrocarbon catabolism on the agar plate in 12 weeks of incubation.

Group No.	Test Bacilli*	Incub. Temp.	Chemically Definded media	LB-spot Color change	Colony formation & color of colonies Early (0 to 4 wks)⇒Late (8 to 12 wks)	No, of colonies per spot***	CFR **** (per million)
1	168-prototr.	28°C	BM1βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
2	168-prototr.	28°C	BM2βTNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
3	WH-0**	28°C	BM1βTNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
4	WH-0	28°C	BM1βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
5	WH-0	28°C	BM2βTNT	Red brown	White colonies ⇒brown col. later	46.4±33.2 (3)	2.02
6	WH-0	28°C	BM2βBen	No change	White colonies ⇒deep brown col. later	52.0±37.0 (3)	2.26
7	WH-0	28°C	BM2βTol	No change	White colonies ⇒deep brown col. later	50.6±35.4 (3)	2.20
8	WH-0	28°C	BM2βBP	No change	White colonies ⇒yellow brown col. later	68.5±33.8 (3)	2.98
9	WH-0	28°C	BM2β(+Nitrite^) TNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
10	WH-0	28°C	BM3βTNT	Light brown	White colonies ⇒brown col. later	14.7±17.8 (3)	0.64
11	WH-0	28°C	BM2β(+Nitrite) TNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
12	WH-0	38°C	BM1βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
13	WH-0	38°C	BM2βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
14	WH-0	38°C	BM2βTNT	Brown	White colonies ⇒brown col. later	3.4 ±3.2 (3)	0.74
15	WH-0	38°C	BM3βTNT	Light Brown	White colonies ⇒brown col. later	4.8 ±2.6 (3)	1.04
16	WH-0	38°C	BM3β(+Nitrite) TNT	Yellow brown	No colony formation	0.0 ± 0.0 (3)	0.00

* Test bacteria in LB broth culture at the late stage of stationary phase was frozen in the deep freezer after the addition of 15% Glycerol. The frozen stocks were removed from the deep freezer and thawed just before the experiment.

** B.subtilis 168 prototrophic transformed with plasmid pTV1Ts was designated as WH-0

*** Number of colonies per bacterial spots was calculated as the mean numbers and standard deviation of colonies from total of 27 spots from 3 plates and expressed in the formula of $m \pm \sigma_{n-1}$ (3 plates).

**** Colony formation rate per million was calculated from the number of colony formation on test medium per that on the TBAB plate with the dilution factor of a million. It was based from the dilution experiment that 1 million dilution of test bacteria WH-0 will form 92 colonies/ml on TBAB plate and that the test bacteria of WH-1 in 1 million times dilution will form 76 colonies per ml on the TBAB plate.

^ Nitrite was added by inclusion of 15.1 mM sodium nitrite (the same amount as sodium nitrate in the medium).

Table 3. Construction of hydrocarbon catabolizing gene-mechanism on B. mycoides (MR-1) by transformation or gene-fusion of the bacilli with plasmid pTV_1Ts. Bacilli were applied to 9 spots on the chemically definded media followed by application of hydrocarbons to 4 evenly-distributed chemical spots for induction of hydrocarbon catabolism on the agar plate in 12 weeks of incubation.

Group No.	Test Bacilli*	Incub. Temp.	Chemically Definded Media	LB-spot Color change	Colony formation & color of colonies Early (0 to 4 wks) ⇒Late (8 to 12 wks)	No, of colonies per spots***	CFR**** (per million)
1	MR-1	28°C	BM1βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
2	MR-1	28°C	BM2βTNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
3	WH-1**	28°C	BM1βTNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
4	WH-1	28°C	BM1β Ben	No change	No colony formation	0.0 ± 0.0 (3)	0.00
5	WH-1	28°C	BM2βTNT	Red brown	White colonies ⇒brown col. Later	7.1 ±4.8 (3)	1.86
6	WH-1	28°C	BM2βBen	No change	White colonies ⇒deep brown col. later	10.6 ±4.3 (3)	2.78
7	WH-1	28°C	BM2βTol	No change	White colonies ⇒deep brown.col. later	10.2 ± 6.8 (3)	2.67
8	WH-1	28°C	BM2ββBP	No change	White colonies ⇒yellow brown col. later	11.0 ±4.5 (3)	2.88
9	WH-1	28°C	BM2β(+Nitrite^) TNT	Pale brown	No colony formation	0.0 ± 0.0 (3)	0.00
10	WH-1	28°C	BM3TNT	Light brown	White colonies ⇒brown col. later	6.4 ± 3.3 (3)	1.60
11	WH-1	28°C	BM3β(+Nitrite) TNT	Yellow brown	No colony formation	0.0 ± 0.0 (3)	0.00
12	WH-1	28°C	BM1β Ben	No change	White colonies ⇒white col. Later	5.2 ± 0.0 (3)	1.35
13	WH-1	38°C	BM2βBen	No change	White colonies ⇒deep brown col. later	5.3 ± 3.8 (3)	1.39
14	WH-1	38°C	BM2βTNT	Red brown	White colonies ⇒brown col. later	3.8 ± 2.7 (3)	0.94
15	WH-1	38°C	BM3βTNT	Brown	White colonies ⇒brown col. later	4.8 ± 2.9 (3)	1.26
16	WH-1	38°C	BM3β(+Nitrite) TNT	Yellow brown	No colony formation	0.0 ± 0.0 (3)	0.00

* Test bacteria in LB broth culture at the late stage of stationary phase was frozen in the deep freezer after the addition of 15% glycerol. The frozen stocks were removed from the deep freezer and thawed just before the experiment.

** MR-1 (B. mycoides) was isolated from Mississippi River and transformed with plasmid pTV_1Ts was designated as WH-1.

*** Number of colonies per bacterial spots was calculated as the mean numbers and standard deviation of colonies from total of 27 spots from 3 plates and expressed in the formula of $m \pm \sigma_{n-1}$ (3 plates)

**** Colony formation rate per million was calculated from the number of colony formation on test medium per that on the TBAB plate with the dilution factor of a million. It was based from the dilution experiment that 1 million dilution of test bacteria WH-0 will form 92 colonies/ml on TBAB plate and that the test bacteria of WH-1 in 1 million times dilution will form 76 colonies per ml on the TBAB plate.

^ Nitrite was added by inclusion of 15.1 mM sodium nitrite (the same amount as sodium nitrate in medium).

Table 4. Construction of hydrocarbon catabolizing gene-mechanism on B. subtilis (168 protrophic) by transformation or gene-fusion of the bacilli with plasmid pLTV1. Bacilli were applied to 9 spots on the chemically definded media followed by application of hydrocarbons to 4 evenly-distributed on chemical spots for induction of hydrocarbon catabolism on the agar plate in 12 weeks of incubation.

Group No.	Test Bacilli*	Incub. Temp.	Chemically Definded media	LB-spot Color change	Colony formation & color of colonies Early (0 to 4 wks) ⇒Late (8 to 12 wks)	No, of colonies per spot***	CFR**** (per million)
1	168-prototr.	28°C	BM1βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
2	168-prototr.	28°C	BM2βTNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
3	WH-2**	28°C	BM1βTNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
4	WH-2	28°C	BM1βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
5	WH-2	28°C	BM2βTNT	Red brown	White colonies ⇒brown col. later	7.1 ± 4.8 (3)	1.42
6	WH-2	28°C	BM2βBen	No change	White colonies ⇒deep brown col. later	13.6± 4.2 (3)	2.72
7	WH-2	28°C	BM2βTol	No change	White colonies ⇒deep brown col. later	11.4± 3.6 (3)	2.27
8	WH-2	28°C	BM2βBP	No change	White colonies ⇒yellow brown col. later	13.2± 3.8 (3)	2.64
9	WH-2	28°C	BM2β(+Nitrite^) TNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
10	WH-2	28°C	BM3βTNT	Light brown	White colonies ⇒brown col. later	3.5 ± 2.1 (3)	0.78
11	WH-2	28°C	BM3β(+Nitrite) TNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
12	WH-2	38°C	BM1βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
13	WH-2	38°C	BM2βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
14	WH-2	38°C	BM2βTNT	Brown	White colonies ⇒brown col. later	3.4 ±3.2 (3)	0.74
15	WH-2	38°C	BM3βTNT	Light Brown	White colonies ⇒brown col. later	3.2 ±2.2 (3)	0.64
16	WH-2	38°C	BM3β(+Nitrite) TNT	Yellow brown	No colony formation	0.0 ± 0.0 (3)	0.00

* Test bacteria in LB broth culture at the late stage of stationary phase was frozen in the deep freezer after the addition of 15% glycerol. The frozen stocks were removed from the deep freezer and thawed just before the experiment.

** B.subtilis 168 prototrophic transformed with plasmid $pLTV_1$ was designated as WH-0

*** Number of colonies per bacterial spots was calculated as the mean number and standard deviation of colonies from total of 27 spots from 3 plates and expressed in the formula of $m \pm \sigma_{n-1}$ (3 plates)

*** Colony formation rate per million was calculated from the number of colony formation on test medium per that on the TBAB plate with the dilution factor of a million. It ws based from the dilution experiment that 1 million dilution of test bacteria WH-0 will form 92 colonies/ml on TBAB plate and that the test bacteria of WH-1 in 1 million times dilution will form 76 colonies per ml on the TBAB plate.

^ Nitrite was added by inclusion of 15.1 mM sodium nitrite(the same amount as sodium nitrate in the medium).

Table 5. Construction of hydrocarbon catabolizing gene-mechanism on B. mycoides (MR-1) by transformation or gene-fusion of the bacilli with plasmid pLTV1. Bacilli were applied to 9 spots on the chemically definded media followed by application of hydrocarbons to 4 evenly-distributed chemical spots for induction of hydrocarbon catabolism on the agar plate in 12 weeks of incubation.

Group No.	Test Bacilli*	Incub. Temp.	Chemically Definded media	LB-spot Color change	Colony formation & color Early (0 to 4 wks)⇒Late (8 to 12 wks)	No, of colonies per spot ***	CFR**** (per million)
1	MR-1	28°C	BM1βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
2	MR-1	28°C	BM2βTNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
3	WH-3**	28°C	BM1βTNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
4	WH-3	28°C	BM1βBen	No change	No colony formation	0.0 ± 0.0 (3)	0.00
5	WH-3	28°C	BM2βTNT	Red brown	White colonies ⇒brown col. later	8.9 ± 9.9 (3)	3.18
6	WH-3	28°C	BM2βBen	No change	White colonies ⇒deep brown col. later	10.4± 3.2 (3)	3.72
7	WH-3	28°C	BM2βTol	No change	White colonies ⇒deep brown col. later	10.2± 3.6 (3)	3.64
8	WH-3	28°C	BM2βBP	No change	White colonies ⇒yellow brown col. later	11.6± 5.8 (3)	4.14
9	WH-3	28°C	BM2β(+Nitrite ^) TNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
10	WH-3	28°C	BM3βTNT	Light brown	White colonies ⇒brown col. later	4.6 ± 2.1 (3)	3.40
11	WH-3	28°C	BM2β(+Nitrite) TNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00
12	WH-3	38°C	BM1βBen	No change	White colonies ⇒white col. later	2.4 ± 2.2 (3)	0.85
13	WH-3	38°C	BM2βBen	No change	White colonies ⇒deep brown col. later	4.3 ± 2.6 (3)	1.54
14	WH-3	38°C	BM2βTNT	Brown	White colonies ⇒brown col. later	4.5 ± 3.6 (3)	1.60
15	WH-3	38°C	BM3βTNT	Light Brown	White colonies ⇒brown col. later	3.9 ± 3.2 (3)	1.39
16	WH-3	28°C	BM3β(+Nitrite) TNT	No change	No colony formation	0.0 ± 0.0 (3)	0.00

* Test bacteria in LB broth was cultured to the stationary phase, and was frozen in the deep freezer after the addition of 15% glycerol. The frozen stocks were removed from the deep freezer and thawed just before the experiment.

** A strain of B.mycoides (MR-1) isolated from the Mississippi River was transformed with plasmid $pLTV_1$ and designated as WH-3

*** Number of colonies per bacterial spots was calculated as the mean number and standard deviation of colonies from total of 27 spots from 3 plates and expressed in the formula of $m \pm \sigma_{n-1}$ (3 plates)

**** Colony formation rate per million was calculated from the number of colony formation on test medium per that on the TBAB plate with the dilution factor of a million. It was based from the dilution experiment that 1 million dilution of test bacteria WH-0 will form 92 colonies/ml on TBAB plate and that the test bacteria of WH-1 in 1 million times dilution will form 76 colonies per ml on the TBAB plate.

^ Nitrite was added by inclusion of 15.1 mM sodium nitrite (the same amount as sodium nitrate in the medium).