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TRANSFORMATION OF PSEUDOMONAS PUTIDA WITH CHROMOSOMAL DNA

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SUMMARY: Auxotrophic cells of P. putida strain PpG1 can be transformed for a number of chromosomal markers. The frequency of transformation is low (10^{-6} - 10^{-7} at optimum DNA concentration), and prior treatment of the cells with $CaCl_2$ is necessary. The optimum concentration of $CaCl_2$ for the induction of competence is variable among recipients. The concentration of DNA required to produce maximal frequency of transformation is high (5 - 10 µg/ml), and only high molecular weight double-stranded DNA appears to be biologically active. Treatment of the transforming DNA with restriction endonucleases and DNase, but not RNase, heating at 100°C and quick cooling, sonication, etc., completely abolishes transformation. The integration of the transforming DNA into the recipient chromosome has been inferred from co-transformation of closely linked genetic characters.

INTRODUCTION: Gene transfer among bacteria is accomplished via three principal modes: transduction, conjugation, and transformation. While gene exchange appears to be common among both gram-positive and gram-negative bacteria, transformation appears to be the principal mode of gene exchange among gram-positive bacteria, such as Bacillus (1), and rare among the gram-negative bacteria. In contrast, gene exchange by plasmid-mediated sexual conjugation is common among gram-negative bacteria such as Escherichia coli and Salmonella typhimurium (2), and appears to be rather infrequent among the gram-positive bacteria. Recently, however, transformation of E. coli and S. typhimurium has been demonstrated with plasmid DNA, provided the cells are treated with $CaCl_2$ to make them permeable to the naked DNA (3,4) and also with chromosomal DNA, provided the E. coli cells additionally lacked ATP-dependent DNase and Exonuclease I (5). Pseudomonas putida is a gram-negative soil bacterium, which has been demonstrated to undergo both transductional (6,7) and conjugational gene exchange (8) with other members of the species.

We have also demonstrated previously that $CaCl_2$-treated cells of P. putida

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can be transformed with both antibiotic resistance and hydrocarbon degradative plasmid DNA to antibiotic resistance and hydrocarbon utilization characters (9).

In this paper, we present some evidence that suggests that $CaCl_2$-treated cells of P. putida can also be transformed with chromosomal DNA, albeit at a low frequency, and such transforming DNA can recombine with the recipient chromosome with replacement of some preexisting segments of the chromosome. A high degree of cotransformation of closely linked markers suggests that transformation might be a useful tool for fine-structure mapping of chromosomal genes in P. putida.

MATERIALS AND METHODS:

Bacterial Strains and Their Propagation. The auxotrophic strains, used as recipients for transformation, are all derived from P. putida strain PpGl. The maintenance and culture conditions have previously been described (6,8). The relevant genotypic properties of these strains are described in Table I.

Isolation of the Transforming DNA. The frequency of transformation varies widely depending upon the method of DNA isolation. For maximal transformation frequency, the donor is grown with L broth overnight on a shaker at 32°C, and the DNA is extracted by gently lysing the cells with lysozyme-EDTA and sarkosyl NL97, essentially as described earlier (10). The NaCl treatment is eliminated to retain maximal amount of chromosomal DNA, which is then banded in a CsCl-ethidium bromide gradient (10). The band corresponding to the chromosomal DNA is then collected, dialyzed against TES buffer overnight, followed by a final dialysis in the TE buffer (10) for about 20 hours. The concentration of the DNA is usually maintained at 50 - 100 µg/ml and is stored at 1 - 2°C in the dark over a drop of chloroform.

Transformation Procedure. The transformation procedure is basically the same as described previously for plasmid DNA (9). The recipient cells are grown overnight in L broth at 32°C on a shaker. A 5% inoculum of the recipient is used to reinoculate into fresh L broth and grown for 3 hours under the same conditions as above. At the end of the 3 hour growth period, the cells are chilled in ice, harvested by centrifugation, and resuspended in half the original volume in 10 mM NaCl. The cells are again harvested, and resuspended in half the original volume in 0.1 M $CaCl_2$, unless otherwise stated. The cells are kept at 0°C for 20 minutes, harvested and resuspended in 1/10 the original volume in 0.1 M $CaCl_2$. For some recipients with 0.3 M or 0.4 M optimal $CaCl_2$, the $CaCl_2$ concentration is maintained the same (0.3 or 0.4 M) throughout the experiment. To 100 µl of $CaCl_2$-treated cells, 50 µl of the chromosomal DNA in Tris-EDTA buffer pH 7.4, were added to give an optimum DNA concentration and kept at 0°C for 1 hour. The cell-DNA mixture is then subjected to a 42°C heat pulse for 2 minutes and chilled in ice for 5 minutes. It is then diluted with 10 times its volume of L broth and grown overnight. Aliquots of the overnight grown cultures were plated on nutrient agar plates for titer, while a portion is centrifuged, suspended in 1/5 the original volume, and 0.1 ml aliquots plated on selective minimal glucose plates supplemented with non-selective amino acids or vitamins. The plates are usually incubated for 2 - 3 days at 32°C before scoring the number of transformants.

RESULTS: The transformability of auxotrophic mutants of Pseudomonas putida strain PpGl by isolated DNA can be seen from the results in Table 2. The ab-

Table 1 Bacterial strains used

Strain	Genotype	Reference/Derivation
P. putida AC30	wt	10
AC587	met601, trpD6111, str, pf-r	13
AC577	trpF316, leu501, str, pf-r	13
AC783	ade1 str	13
AC10	met1	10
AC749	arg11, str, pf-r	13
AC789	met2, his301, str, pf-r	NTG mutagenesis

Gene symbols: str, streptomycin resistance; pf-r, resistance to phage pf16; met, methionine biosynthesis; trp, tryptophan biosynthesis; leu, leucine biosynthesis; ade, adenine biosynthesis; arg, arginine biosynthesis; his, histidine biosynthesis; NTG, N-methyl-N'-nitro-N-nitrosoguanidine

sence of any transformants, when the donor DNA carried the same mutational alleles as the recipient, clearly suggests that the exogenous DNA is responsible for the repair of the recipient mutations. The transformants are stable and do not appear to lose the donor markers, indicating the haploid nature of such transformants.

In order to see if transformation of genetic characters in P. putida is limited to a few loci, or extends to diverse portions of the genome, a number of auxotrophic recipients harboring mutations that map all over the chromosome were treated with DNA isolated from wild type or other auxotrophic donors. The results of such an experiment are shown in Table 3. Although the frequency is low (10^{-6} - 10^{-7}/ viable cell), a number of recipients can be transformed to prototrophic characters with the wild type DNA. In addition, other markers, for which accurate map position have not been determined, can also be repaired by transformation with such DNA.

That the repair of the auxotrophic mutations is in fact due to the presence of the exogenous DNA can be further demonstrated from the results of Table 4. In these experiments, the transforming DNA was subjected to treatment with DNase

Table 2 Transformation of P. putida PpG1 with Chromosomal DNA

Donor DNA (genotype)	Number of Transformants		
	adel$^+$	trpF316$^+$	leu501$^+$
None	0	1	1
trpF316 leu501	732	0	1
trpD6111 met601	1850	282	359

$CaCl_2$-treated cells of AC783 and AC577 were treated with chromosomal DNA extracted from AC577 and AC587 as described under MATERIALS AND METHODS. The concentration of DNA used was about 20 µg/ml, and the number of transformants corresponds to about 2×10^9 cells.

and restriction endonucleases, RNase, sonication, etc., and the effect of the treated DNA on the frequency of transformation studied. As can be seen, treatment of the DNA with DNase and Eco R1, but not RNase, abolishes transformation. Similarly, denaturation of the DNA by heating it in a boiling water bath for 10 minutes followed by quick chilling in ice and sonication for 10 minutes, greatly reduces the frequency of transformation. It is thus clear that the presence of double-stranded high molecular weight DNA is essential for transformation of chromosomal markers.

The effect of varying DNA concentration using cells treated with optimal concentration of $CaCl_2$ (0.1 M) has also been studied. The curve is linear up to about 8 µg/ml of DNA but shows variable peaks above this concentration. In general, a saturating concentration of DNA isolated by the procedure described by Marmur (11) produces a lower number of transformants than the DNA isolated by the dye-CsCl buoyant density gradient.

The linkage relationships among genes that appear to be linked by transduction can also be determined by transformation. This is shown by the results in Table 5. Thus, met601, leu501, and trp316 have previously been shown to be linked with a cotransfer index of 0.7 to 0.8 (12), and the gene cluster also appears to enter at nearly 26 minutes during interrupted mating (13). The recovery of a large number of Met$^-$ recombinants during transformation of AC577

Table 3 Transformability and map position of some chromosomal markers in P. putida

Recipient strain	Genotype	map position (min)	Transformation Frequency
AC10	met1	13	4×10^{-8}
AC749	arg11	17	1.1×10^{-6}
AC577	trpF316	25	5.1×10^{-7}
	leu501	25	6.5×10^{-7}
AC587	met601	25	3.2×10^{-8}
	trpD6111	35	1.5×10^{-7}
AC783	ade1	26	2.3×10^{-6}
AC789	his301	ND*	3.4×10^{-6}

The transformation frequency is expressed as the number of prototrophic recombinants relative to the total number of viable cells. The transformation frequency is not optimum, since cells were treated with 0.1 M $CaCl_2$, which is not optimum for some recipients, and DNA concentration (about 6 µg/ml) may not have been the optimum for others.

*ND - not determined; for details of map position see ref. 13

(trp316 leu501) with the DNA isolated from AC587 (met601 trpD6111) suggests that the donor DNA is physically integrated with the recipient chromosome. In the reciprocal cross, selection for Met^+ transformants on glucose-minimal plates supplemented with tryptophan-leucine produces a large number of transformants that carry the donor leu501 allele. In contrast, no transfer of leu501 allele takes place, when selection is made for $trpD^+$ on glucose-minimal plates supplemented with leucine-methionine. The trpD6111 locus is known to be transductionally unlinked with trp316 leu501 met601 cluster (12) and enters at around 35 minutes during interrupted mating (13). The lack of cotransfer of leu501 with trpD6111, therefore, confirms the earlier observations that these two loci are too far apart to undergo simultaneous integration during transformation with the chromosomal DNA.

Further proof that the transforming DNA is integrated into the recipient chromosome is obtained by infecting the transformants harboring the donor met601 mutation ($leu501^+$ met601 trpF316 transformant, Table 5) and transducing

Table 4 Effect of various treatments of the transforming DNA

Treatment of DNA	Number of transformants	
	Leu^+	Trp^+
No treatment	88	68
DNase (5 µg/ml)	4	1
RNase (50 µg/ml)	119	43
Eco R1 (10 units/ml)	5	5
Sonication	3	0
Denaturation (100°, 10 min)	1	6
No DNA	1	4

DNA (6-8 µg/ml final concentration) was from AC587, and AC577 was used as a recipient. The transformants were selected on minimal glucose plates with trypophan-methionine (for leu^+ selection) or leucine-methionine (for trp^+ selection) as supplements. About $2x10^9$ cells were plated to produce the number of transformants scored. Treatment with DNase was at $37^{\circ}C$ for 30 min in presence of 10 mM $MgCl_2$, while treatment with RNase was at $37^{\circ}C$ for 1 hr. Sonication was done for 5 min with a Raytheon, model DF101 sonic oscillator in the cold. Denaturation was done by heating the DNA in TE buffer in a boiling water bath for 10 min, followed by chilling in an ice bath.

a recipient with leu501 mutation to leu^+. About 58% of such leu^+ transductants also inherit the donor met601 and trpF316 mutations simultaneously, suggesting that these two mutations are present on a single fragment of the chromosome in the original transformant.

DISCUSSION: The ability of the wild type P. putida strain PpG1 cells to be transformed for chromosomal characters by naked DNA again points out to the versatility of a soil microorganism to develop efficient modes of gene exchange for survival in a highly competitive environment. Undoubtedly, the frequency of such exchange is low, and the presence of optimum concentrations of cations is necessary. Transformation may, therefore, be an infrequent mode of genetic exchange among P. putida. In case of enteric bacteria such as E. coli, the presence of the recB recC gene product, ATP-dependent DNase, and to a lesser extent that of the sbcB gene product, exonuclease I, prevents chromosomal transformation (5). Since double-stranded circular DNA (with or without nicks) is

Table 5 Co-transformation of closely-linked markers

DNA (genotype)	Recipient (genotype)	Selected Marker	No. NSM^+/Total Met^-	No. NSM^+/Total Leu^-	Linkage (%)
met601 trpD6111	leu501 trpF316	leu^+	62/93		66
"	"	trp^+	89/97		91
leu501 trpF316	met601 trpD6111	met^+		60/74	81
"	"	trp^+		0/109	0

$CaCl_2$-treated cells of AC577 and AC587 were treated with DNA extracted from AC587 and AC577 as described under MATERIALS AND METHODS. The total number of cells plated were almost 10^9, and NSM^+ denotes the number of cells that were positive for the non-selective marker.

immune to attack by the ATP-dependent DNase, while linear molecules are extensively degraded by this enzyme, it has been concluded that the lack of transformation of chromosomal characters in wild type E. coli cells is due to extensive damage of the incoming linear chromosomal DNA fragments by the DNase (5). It would be interesting to find out if wild type P. putida cells lack these enzymes. Preliminary assay for the presence of ATP-dependent DNase in wild type, and a MMS-sensitive mutant of P. putida does suggest the presence of this enzyme in the wild type cells (S. Basu, personal comm.). If this is true, it would be interesting to find out if the transformation frequency in P. putida can be greatly increased by isolating mutants lacking these two enzymes.

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