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Evidence for an Active Role of Donor Cells in Natural Transformation of *Pseudomonas stutzeri*

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The transfer of chromosomal genes in a cell mat of *Pseudomonas stutzeri* was ca. 10^3 times more efficient per microgram of DNA if DNA was added as a constituent of intact donor cells rather than as a solution. Such intact cell-mediated transfer appears to depend on cell contact. It is independent of the presence of plasmids in donor strains and is DNase I sensitive, thus fitting the usual definition of transformation. It is bidirectional: cells of either strain in a transformation mixture served as the donor and recipients. The donor function in cell contact transformation was inhibited by nalidixic acid but was unaffected by rifampin and streptomycin at growth-inhibiting concentrations. Concentrations of nalidixic acid sufficient to inhibit donor function completely had no effect on the ability of nalidixic acid-resistant recipients to take up DNA from solution. These experiments suggest that certain cells donate DNA to others in the cell mat: they argue against the hypothesis that the function of donor cells is merely cell lysis.

Transformation, the first mechanism of bacterial genetic exchange discovered (10), has been intensively investigated in a number of species (12, 14, 19, 22, 23). Whereas the mechanism for DNA uptake has been characterized in both gram-positive and gram-negative bacteria (19), the mechanism whereby DNA becomes available to recipient cells in a population is less well understood. Several models for DNA release by donor cells have been proposed, including cell lysis (8) and active excretion (17, 21). Extracellular DNA has been detected in cultures of a wide variety of bacteria, including *Bacillus subtilis* (8), *Streptococcus pneumoniae* (16), and *Neisseria meningitidis* (6). In at least some species, extracellular DNA appears to be retained on the surface of cells rather than being expelled into the medium (8, 20).

It has been established that the release of DNA from *B. subtilis* involves active replication of the chromosome (15). Inhibition of DNA synthesis by 6-(*p*-hydroxyphenylazo)uracil resulted in rapid cessation of DNA release. With synchronized germinating spores, genetic markers were transformed sequentially from an origin at or near the origin of chromosome replication (4). The chromosome in some bacteria is attached to mesosomes on the inner surface of the cytoplasmic membrane, and replication may be associated with this attachment site (13). It is clear that for both *B. subtilis* (9) and *Escherichia coli* (18) newly replicated DNA is membrane attached. Furthermore, the release of DNA from donor *B. subtilis* cells closely follows the initiation of chromosome replication, and the port for DNA exit may be the chromosome-membrane attachment structure (4).

These studies suggest that the contribution of DNA for transformation may require active participation by the donor cell through coupling of DNA replication and DNA extrusion. We report here results of experiments that implicate donor cells as playing an active, DNA synthesis-dependent role in the natural transformation of *Pseudomonas stutzeri* (5).

MATERIALS AND METHODS

Bacterial strains. The strains of *P. stutzeri* used in this study (Table 1) were all derived from JM300, a smooth variant of a natural soil isolate, JM299(5). RK2 was introduced into *P. stutzeri* by conjugation from C600 (our laboratory no. JL2913), a strain of *E. coli* with the genotype *leu thi thr*.

Media and growth conditions. The synthetic medium used was a basal salts medium devised by B. A. Bryan with 30 mM succinate as a carbon source (5). When required, amino acids were added to a final concentration of 50 μg/ml. Enriched medium was Luria-Bertani (LB), containing (per liter), 10 g of tryptone (Difco Laboratories), 5 g of yeast extract (Difco), 5 g of NaCl, and 1 g of glucose, modified by the addition of 1 mM $MgSO_4$ and 10 mM Tris-hydrochloride (pH 7.5). Unless stated otherwise, antibiotics were added to media at the following final concentrations (micrograms per milliliter): kanamycin sulfate (Sigma Chemical Co.) 50; streptomycin sulfate (Sigma), 100; nalidixic acid (Sigma), 50; and rifampin (Calbiochem), 50. Solidified media contained 2% (wt/vol) Bacto-Agar (Difco). Liquid cultures were grown under constant aeration in a

TABLE 1. Strains of *P. stutzeri*

Strain	Genotype
JM302	*his-1*
JM303	*trp-1*
JM493	*rif-12 trp-1*
JM501	*trp-1*(RK2)
JM506	*rif-12 trp-1*(RK2)
JM509	*nal-9 rif-12 trp-1*
JM512	*rif-12 str-7 trp-1*

rotary shaking water bath (New Brunswick Scientific Co.). Growth of liquid cultures was monitored with a Klett-Summerson colorimeter with a red filter. All incubations were at 37°C.

Isolation and purification of DNA. LB broth was inoculated with an overnight culture to a density of 20 Klett units. Cultures were incubated until a density of 100 Klett units was obtained. Then the cells were harvested by centrifugation at 10,400 × *g* for 15 min and washed with TES (50 mM Tris-hydrochloride [pH 8.0], 5 mM EDTA, 50 mM NaCl). The cell pellet was suspended in 30 ml of spheroplast buffer (25% sucrose, 50 mM Tris-hydrochloride [pH 8.0]); 30 mg of lysozyme (US Biochemicals) was added, and the cell suspension was held on ice for 30 min. Then, 3.0 ml of 0.25 M EDTA was added, and the cell suspension was incubated for 15 min on ice; 3.3 ml of 10% Sarkosyl in TES was gently mixed in by swirling. The lysed cell suspension was treated with 10 μl of RNase I (Sigma [5 mg/ml preheated to 65°C for 10 min]), and incubated at room temperature for 30 min. Then, 1.5 ml of pronase I (Calbiochem [10 mg/ml, predigested at 37°C for 1 h]) was added, followed by incubation at 37°C for 30 min. The cell lysate was then extracted twice with equal volumes of phenol-chloroform (1:1), followed by TES-saturated diethyl ether until clear. DNA precipitated by the addition of a 2× volume of 95% ethanol (−20°C) was spooled onto a glass rod, dissolved in 1 to 2 ml of TES, and dialyzed twice against 1 liter of TES.

DNA was determined by a diphenylamine Dische assay (2) with salmon sperm DNA as the standard. DNA solutions were sterilized by heating for 2 h at 65°C.

Transformation with purified DNA. Recipient cells were grown by inoculating 25 ml of LB broth with an overnight culture to 20 Klett units and incubating at 37°C with shaking to 130 to 140 Klett units. One milliliter of the late exponential culture was filtered dropwise through a membrane filter (diameter, 47 mm; pore size, 0.45 μm) to give a cell mat ca. 1 cm in diameter. The filter was placed cell side up on the surface of a dry LB broth agar plate, and appropriate volumes of purified DNA sheared to optimal size for transformation (5) were added evenly over the surface of the cell mat. Plates were incubated upright at 37°C for 16 h. The cells were suspended from the filters in 10 ml of minimal succinate containing 0.2% LB broth by gentle shaking for 20 min at room temperature on a Junior Orbital shaker (Labline). Cell suspensions were serially diluted in the same medium, and 0.1 ml of appropriate dilutions was plated on selective media to count recipients and transformants.

Transformation by cell contact. Recipient cells were prepared as described above. Donor cells were grown similarly to 70 Klett units. Donor and recipient cultures were mixed 1:10, and 1.0 ml of the cell mixtures was immobilized on membrane filters, incubated, and then plated as was done in transformation experiments with purified DNA.

DNase I treatments. A 2-mg/ml (~3,000 U/ml) solution of DNase I (Sigma) in 4.2 mM $MgCl_2$ was filter sterilized and stored on ice; 0.1 ml of this was pipetted directly over the cell mat, and the plates were gently tilted to distribute the enzyme evenly over the surface of the mat.

RESULTS

Simultaneous transfer of genes by transformation and conjugation. In experiments designed to evaluate the ability of the IncPl plasmid RK2 (11) to mobilize chromosomal genes of *P. stutzeri*, we noticed that cofiltering donors and recipients (i.e., direct cell contact) stimulated a significant transfer of chromosomal as well as plasmid-encoded genes; the latter were transferred at a higher frequency than the former (Table 2). This pattern of transfer resembled conjugal chromosome mobilization. However, we have previously shown that *P. stutzeri* undergoes natural transformation (5), so control matings in the presence of DNase were done to identify the contribution of transformation to the process. They showed that the transfer of chromosomal genes was completely sensitive to DNase treatment, whereas the transfer of plasmid genes was completely resistant (Table 2). Inhibition of gene transfer by exogenous DNase I was taken as proof that DNA was extracellular at some point in the process; such a process meets the usual definition of transformation. Thus, cofiltration appeared to stimulate the transfer of plasmid genes by conjugation and chromosomal genes by transformation.

A requirement for cell contact in this direct transformational transfer of chromosomal genes was established by comparing frequencies of transformation between cells immobilized on filters with frequencies of transformation between cells suspended in the same liquid medium. On filters, frequencies of ca. 10^{-4} transformants per recipient were obtained; in liquid, the frequencies were below the level of detection. Increasing the surface area in the liquid transformation by the addition of sterile glass beads resulted in a detectable intermediate frequency of transformation of about 10^{-7} transformants per recipient (data not shown).

To determine whether the RK2 plasmid facilitated the transformational transfer of chromosomal genes, isogenic strains differing only in the presence or absence of RK2 were compared as donors (Table 3). Although the frequency of transformation differed with the particular marker studied (probably because the pairs of experi-

TABLE 2. Effect of DNase I treatment on the transfer of plasmid- and chromosome-encoded genes

Donor	Recipient	Marker transferred		Frequency of transfer[a]	
		Location	Designation	Without DNase	With DNase[c]
JM501	JM302	Plasmid[b]	Km^r	2.3×10^{-1}	2.7×10^{-1}
JM501	JM302	Chromosome	*his-1*	2.2×10^{-5}	$<10^{-8}$
JM506	JM302	Chromosome	*rif-12*	2.0×10^{-5}	$<10^{-8}$

[a] Recombinants per recipient.
[b] RK2.
[c] A total of 0.1 ml of a 2-mg/ml sterile solution of DNase I in 4.2 mM $MgCl_2$.

ments were done at different times with cells in different physiological states), their frequency of transfer was completely unaffected by the presence of RK2. Thus, such cell contact transformation (as we have termed the DNase-sensitive transfer of chromosomal genes that occurs in cell mats) is not mediated or facilitated by the conjugative plasmid RK2.

To determine whether cell contact transformation is mediated in a polarized manner by an undetected plasmid or by some other factor, a cross was made between a histidine auxotroph (JM302) and a tryptophan auxotroph that was resistant to rifampin (JM493). Thus, in a single mating mixture, the transfer of each of the three genes could be independently scored, and on the assumption that rifampin resistance is not cotransformed with either histidine or tryptophan auxotrophs by cell contact because these auxotrophs are not cotransformed with detectable frequency by a solution of DNA (data not shown), the direction of transfer could be inferred. Both strains in the mixture donated and received genes at approximately equal frequencies (Table 4).

Comparison of the efficiencies of transformation elicited by the addition of intact cells and of DNA in solution. Per unit of DNA, transformation by cell contact was markedly more efficient than transformation elicited by the addition of a solution of purified DNA to a mat of recipient cells. The frequency of transfer of both the *trp-1* and *rif-12* alleles reached a maximum when ca. 1 μg of purified DNA was added to mats of recipient cells on membrane filters. The addition of larger amounts of DNA (up to 10 μg) had no further effect on the frequency of transformation. At saturating levels of purified DNA (2 μg), the frequency of transfer of each marker was ca. 10^{-7} per recipient. However, 10^2- to 10^3-fold-higher frequencies of transfer were obtained if DNA was added as intact cells rather than as a solution (Table 5).

The ability of intact cells to produce frequencies of transformants higher than those that could be obtained from saturating levels of DNA in solution suggested a fundamental difference in the two processes. To compare efficiencies of transfer at subsaturating levels of DNA, paired transformational crosses were made in which equivalent amounts of DNA were added to the mat of recipient cells in the form of soluble DNA and as a constituent of intact donors (Table 6). When 0.26 μg of DNA was added as intact cells, the frequency of transformation was 7.0×10^{-5}; when the same amount of DNA was added as a solution, the frequency of transformation was 10^3-fold less.

Active role of donor cells in natural transformation. Comparison of frequencies of transformation by cell contact and by DNA in solution established a significant difference in efficiency per unit of DNA. This difference suggested that the intact donor cell might play an active role in the transformation process by providing DNA in a form that was more readily incorporated by the recipient. In an attempt to characterize this process, specific biosynthetic functions of the

TABLE 3. Effect of the presence of a conjugative plasmid (RK2) on the transfer of chromosomal markers[a]

Donor	Plasmid present	Marker transferred	Frequency of transformation[b]
JM303	−	*his-1*	2.0×10^{-3}
JM501	+	*his-1*	3.0×10^{-3}
JM493	−	*his-1*	2.3×10^{-5}
JM506	+	*his-1*	4.0×10^{-5}
JM493	−	*rif-12*	2.7×10^{-6}
JM506	+	*rif-12*	1.1×10^{-6}

[a] Recipient, JM302.
[b] Recombinants per recipient.

TABLE 4. Bidirectional nature of the transfer of various markers between JM493 and JM302

Direction of transfer[a]	Marker transferred	Frequency of transfer[b]
JM493 to JM302	*rif-12*	2.4×10^{-5}
JM493 to JM302	*his-1*	3.7×10^{-5}
JM302 to JM493	*trp-1*	3.4×10^{-5}

[a] Inferred from the probability of transfer of one or two genes (see text).
[b] Transformants per recipient.

TABLE 5. Comparison of the frequency of transformation by soluble DNA and cell contact

Donor	Recipient	Marker transferred	Frequency of transformation[a]	
			Soluble DNA[b]	Cell contact[c]
JM302	JM493	*trp-1*	8.0×10^{-7}	4.1×10^{-4}
JM493	JM302	*rif-12*	3.0×10^{-7}	2.2×10^{-5}

[a] Transformants per recipient.
[b] Addition of 2 μg of DNA.
[c] Mixture of donor to recipient, 1:10.

donor were selectively inhibited by various antibiotics to which donor cells were sensitive and recipient cells were resistant. Thus, the effect of inhibition of the synthesis of protein, RNA, and DNA on donor function could be studied (Table 7). The inhibition of protein synthesis by streptomycin, and of RNA synthesis by rifampin, at concentrations that were growth inhibiting caused no detectable decrease in transformation frequency. However, the inhibition of DNA synthesis by nalidixic acid decreased the frequency below detectable levels (Table 7).

To ensure that the inhibition of transformation by nalidixic acid was not being exerted through a subtle effect of the antibiotic on the resistant recipient population, its effect on transformation of the same recipient population by purified DNA was measured. This frequency was found to be completely unaffected by nalidixic acid (Table 8). When purified DNA and intact donor cells were added together, nalidixic acid decreased the transformation frequency by ca. 10^2-fold, to the level seen with identical amounts of soluble DNA in the absence of donor cells. Thus, in a single transforming mixture, nalidixic acid appeared to inhibit completely the component of transformation attributable to intact donor cells without affecting the component attributable to DNA in solution. Nevertheless, both the intact cell component and the soluble DNA component were sensitive to DNase I.

DISCUSSION

Attempts to develop a conjugal system of gene transfer in *P. stutzeri* by mobilization of chromosomal genes with the IncP1 plasmid RK2 consistently resulted in distinctly different frequencies of transfer of plasmid and chromosomal markers (Table 2) when donor and recipient strains were immobilized together on membrane filters. The transfer of chromosomal genes occurred at a low frequency and was DNase I sensitive; the transfer of plasmid genes occurred at a high frequency and was insensitive to DNase I. Thus, the transfer of chromosomal genes occurred by a process fitting the usual definition of transformation and plasmid genes by conjugation.

Although natural transformation has been reported in *P. stutzeri* (5), its occurrence in the thin mat of cells on a membrane filter to which no exogenous DNA had been added was unexpected because cell death was undetectable (data not shown), and therefore cell lysis must have been slight. Actual cell contact was found to be essential for intact cells to act as donors in a transformational cross. We presumed that cell contact in the mat might stimulate an active release of DNA from certain cells; i.e., the process that we have termed cell contact transformation might involve an active role of certain donor cells in the population in releasing DNA for transformation. Subsequent experiments showed that cell contact transformation occurred independently of the presence of conjugative plasmids in the donor strain (Table 3) and was bidirectional: cells of each of the two strains released DNA and took it up from cells of the other strain (Table 4). The uptake of DNA from cells in a mat is a markedly more efficient process per unit of DNA than is the uptake of DNA from a solution added to the mat. DNA added in the form of intact cells yielded about 10^3-fold more transformants than did an equal amount of DNA added in solution (Table 6). The greater yield of transformants from cell contact transformation probably is not a simple consequence of the cellularly released DNA being more concentrated than that added from solution, because intact cells produced more transformants than did saturating concentrations of exogenously added DNA (Table 5). That the donor cell plays an active role in cell contact transformation is supported by the observation that its role can be inhibited completely by nalidixic acid but not by rifampin or streptomycin (Table 7); presumably, the ability to synthesize DNA is essential if a cell is to act as a donor in cell contact transformation.

Our experiments, although strongly suggestive, do not offer proof that the DNA causing transformation in cell mats is released from certain donor cells by an active process stimulated by cell contact. Possibly, DNA released by cell lysis has special properties making it many

TABLE 6. DNA dependency of yield of transformants

Method of transformation	Total DNA added (μg)	Frequency of transformation[a]	Relative frequency
Soluble DNA	0.25	6.0×10^{-8}	1
Cell contact	0.26[b]	7.0×10^{-5}	1.7×10^3

[a] Transformants per recipient.
[b] The DNA content of the donor population of 5.2×10^7 donor cells.

TABLE 7. Effect of various inhibitors on transformation by cell contact[a]

Inhibitor	Concn (μg/ml)	Site of inhibition	Relative frequency of transformation[b]
Streptomycin[c]	100	Protein synthesis	1.8
	1,000		0.97
Rifampin[d]	250	RNA synthesis	2.7
Nalidixic acid[d]	50	DNA synthesis	$<10^{-4}$
	100		$<10^{-4}$

[a] Donor for all transformations, JM302. Gene transferred, *trp-1*.
[b] Frequency of transformation in the presence of inhibitor ÷ frequency of transformation in the absence of inhibitor. Frequency of transformation in the absence of inhibitor = 1.0×10^{-4}.
[c] Recipient, JM512.
[d] Recipient, JM509.

times more active than purified DNA. If so, this special property must be something other than concentration (see above) or molecular weight because the exogenous DNA was at a previously determined optimal molecular weight (5). Nalidixic acid conceivably could inhibit donor function by preventing lysis rather than DNA synthesis, but we have been unable to detect any differences in lysis (as indicated by high-pressure liquid chromatography analysis of amino acids in the acid-hydrolyzed supernatant) between a culture inhibited by nalidixic acid (which inhibits donor function) and one not exposed to the antibiotic (data not shown). It might also be argued that recipient cells take up DNA from solution by a different mechanism than the one by which they take up DNA from intact cells. If so, transformation by purified DNA would be an invalid index by which to distinguish the effect of nalidixic acid on donor and recipient function. However, the results presented in Table 8 argue strongly against the possibility that recipient cells take up purified cell-donated DNA by different mechanisms. Purified DNA added to a cell mat yielded transformants at a frequency of about 10^{-6}; cell contact transformation done in the presence of the same amount of added DNA yielded about 10^{-4}. Nalidixic acid reduced the combined frequency to that from purified DNA alone, and DNase eliminated the contribution of both. Thus, DNA released from cells and DNA added as a solution enter a common DNase-available pool. It is difficult to imagine how recipient cells can distinguish between the two sources of DNA that supply the pool.

Studies on transformation have usually focused on the elaborate process by which recipient cells take up DNA from solution. One is left to suspect that in nature the supply of DNA for transformation is dependent on the occasional random lysis of certain cells. It seems unlikely that this is the case. Recipient function is a highly evolved process involving the participation of at least 10 gene products in *S. pneumoniae* (19) and even special recognition sequences in the DNA of *Haemophilus influenzae* (7)—quite elaborate mechanisms to be dependent on random lysis of cells as a source of DNA. Further, cell lysis and development of competence by recipients are not temporally synchronized, the former being more prevalent in stationary cultures and the latter generally occurring during exponential growth, and one would imagine that the half-life of DNA in DNase-abundant natural environments is brief (3).

The extent of the role played by cell contact in natural transformation remains unknown. A fundamental question is to what extent surface contact actually elicits cell-to-cell transformation. Furthermore, during *B. subtilis* transforma-

TABLE 8. Effect of nalidixic acid and DNase I on transformation by soluble DNA and cell contact

Method of transformation[a]	DNase I[b] present	Nalidixic acid[c] present	Frequency of transformation[d]
Soluble DNA	−	−	2.65×10^{-6}
Soluble DNA	−	+	3.92×10^{-6}
Soluble DNA	+	−	$<10^{-8}$
Soluble DNA	+	+	$<10^{-8}$
Cell contact + soluble DNA	−	−	1.44×10^{-4}
Cell contact + soluble DNA	−	+	1.87×10^{-6}
Cell contact + soluble DNA	+	−	$<10^{-8}$
Cell contact + soluble DNA	+	+	$<10^{-8}$

[a] For soluble DNA, 10 μg of purified JM302 DNA was added to the recipient (JM509) spot. For cell contact, whole cells of JM302 were mixed with the recipient (JM509) plus 10 μg of purified JM302 DNA.
[b] A total of 0.1 ml of a 2-mg/ml solution of DNase I in 4.2 mM $MgCl_2$ was added directly over the cell spot.
[c] Amount, 50 μg/ml.
[d] Transformants per recipient.

tion, cell contact is suggested as being responsible for protection of DNA, for there is no stage at which the DNA is sensitive to exogenous DNase (15). However, in *P. stutzeri*, cell contact transformation proceeds without protection of the DNA from DNase. Clearly, the mechanisms are not identical. (Indeed, it is sensitivity to DNase that has been the hallmark of transformation as it is usually characterized.) This distinction is not limited to a difference between gram-positive and gram-negative species. Albritton et al. (1) recently described a process of chromosomal DNA transfer in *H. influenzae* involving cell contact that is unaffected by the development of competence and is only partially sensitive to DNase. Thus, as in *B. subtilis*, the *H. influenzae* process appears to be different from either classical transformation or conjugation but is reminiscent of some aspects of both mechanisms (1). Elucidation of the function of the donor cell and the role of cell contact in the process in each of these species promises to be interesting. The present study presents evidence for an active role of donor cells in natural transformation of *P. stutzeri*, but the nature of the process and the stimuli that trigger it remain unknown.

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