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Conditions for Natural Transformation of *Ralstonia solanacearum*

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The development of competence allowing natural transformation of *Ralstonia solanacearum* was found to occur during exponential growth and not in response to any excreted factors. Linear DNAs were effectively integrated by recombination requiring a minimum of 50 bp of homologous DNA. Therefore, DNA from other genera and species were ineffective.

In the last decade, transformation has been investigated extensively at the molecular and physiological levels for some naturally transformable bacteria that are considered models. These include *Bacillus subtilis* (8), *Neisseria gonorrhoeae* (27), *Acinetobacter calcoaceticus* (5), *Pseudomonas stutzeri* (4), and *Haemophilus influenzae* (11). In parallel, ecological investigations were devoted to determining whether competence development can be achieved under natural conditions (22), particularly in terrestrial ecosystems (14, 21). This motivated our investigations of *Ralstonia solanacearum*, a bacterium previously described as being naturally transformable (3) which is also a pathogen for numerous plants (10). The first step of our work was to investigate the in vitro transformation conditions and, particularly, parameters for development of competence.

Evidence for a transformation mechanism and influence of growth conditions. *R. solanacearum* GMI1000 (17) was transformed on plates by the method of Boucher et al. (3) with the following modifications. Cells were grown in minimal medium (MM; one-quarter-strength M63 medium [16] supplemented with glucose [2 g · liter^{-1}] and glycerol [4 ml · liter^{-1}]) to an optical density at 580 nm of 0.8 (corresponding to 5.0×10^8 cells ml^{-1}). Fifty microliters of this cell suspension was directly mixed with 10 ng (5 μl) of donor DNA of strain GMI1004 (resistant to streptomycin at 200 μg · ml^{-1}), which is a spontaneous mutant of strain GMI1000. The resulting suspension was used to inoculate polycarbonate membranes deposited on the surface of solid MM (MMG) and incubated for 48 h at 28°C (13). Bacterial cells were then harvested from the membrane surface and resuspended in 5 ml of MM, and aliquots were used to inoculate rich medium BG (3) containing the appropriate antibiotics. Each transformation experiment was done at least in triplicate and included determination of the spontaneous mutation rate by plating of nontreated cells on selective media.

In agreement with data acquired with other naturally competent bacteria (14), we found that limiting growth conditions such as those provided by MM permitted a higher transformation efficiency (4×10^{-7}) than did the richer peptone broth medium B (3) (2×10^{-8}) (Table 1). Incubation of cells and DNA for 48 h at 28°C in liquid medium B and MM did not produce a detectable number of transformants (Table 1). Application of standard transformation protocols, including the plating of *R. solanacearum* cells and donor DNA on MMG, resulted in the highest rate (4×10^{-7}). For these experiments, the growth and plating medium compositions were identical. For this strain, our results indicate clearly that only a small percentage of the cultures can be efficiently transformed, unlike transformation of *Azotobacter vinelandii* or *B. subtilis* (19).

Development of the competent stage. In the course of our study, we hypothesized that a particular physiological state of the cells is required to allow transformation to proceed. It has to be kept in mind that in all naturally transformable bacteria, with the only known exception of *N. gonorrhoeae* (29), transformation requires induction of the competent state. Investigation of the nature of the mechanism in a bacterium such as *R. solanacearum* was necessary to determine whether the soil or the plant could provide the conditions required for competence development. This was done by harvesting cells at different stages of growth (liquid MM) before the standard transformation protocol was carried out. To avoid biases related to variability of cell density, volumes of the growing bacteria were adapted to provide a constant number of cells (2.5×10^7) which were subsequently treated for transformation. Standardization of cell density was achieved by concentration or dilution with the culture supernatant. Results shown in Fig. 1 clearly indicate that transformation is related to the development of a competent state, depending on the physiological state of the cells. Only cells in exponential growth achieved the competent state, which declined rapidly during the log phase. Under the experimental conditions used in this study, the maximum transformation frequency (4×10^{-7}) was detected in bacteria harvested at the inflection point of the curve as evaluated by an optical density at 580 nm of 0.8. Moreover, the rate of resistant colonies did not differ from the spontaneous mutation rate when cells harvested before the exponential growth phase were incubated with the growth medium of cells having reached the exponential growth or stationary phase (results not shown). This suggests that induction of the competent stage cannot be related to excretion and accumulation in the surrounding medium of a competence factor but depends only on the physiological state of the cells. According to these results and data from the literature (30), it can be hypothesized that plant tissues, by allowing the cells to develop and multiply extensively, would provide more favorable conditions for competence development of *R. solanacearum* than an oligotrophic bulk soil.

Kinetics of DNA uptake. Another crucial factor that allows transformation to occur in the environment is the speed at which the DNA released by cells is internalized by competent

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TABLE 1. Effect of medium composition on transformation of *R. solanacearum*

Growth medium	Transformation frequency[a]
B	ND[b]
BG	$1.95 (\pm 1.1) \times 10^{-8}$
MM	ND
MMG	$4.06 (\pm 0.79) \times 10^{-7}$

[a] Streptomycin-resistant transformants were selected on media containing streptomycin at 200 mg · ml^{-1}. The viable counts were determined on BG plates in parallel. The transformation frequency was calculated as the number of transformed cells divided by the total viable cell count. Standard errors from replicate experiments are in parentheses. The rate of spontaneous mutation to streptomycin resistance was 3.75×10^{-10}.

[b] ND, transformation not detected (frequency of $\leq 3.75 \times 10^{-10}$).

recipient cells. Kinetics of DNA uptake were defined by subjecting donor DNA and recipient cells to DNase I treatment (20) after incubation on MMG for periods of 0 to 48 h. In laboratory experiments, treatment of DNA with DNase I results in complete degradation of nucleic acid molecules as evaluated by electrophoresis of aliquots on agarose gels (results not shown). It was found that internalization did not proceed instantly, since no transformant was detected for a cell-DNA contact time of less than 10 min. However, the mechanism occurred rapidly (in less than 3 h) with no further increase in transformation efficiency (Fig. 2). However, an incubation time of 48 h permitted the easiest protocol for determination of transformation efficiency and was consequently maintained in the standard transformation method.

Influence of DNA concentration and molecule size on transformation efficiency. A consequence of the presence of nucleases in the environment is that competent bacteria may be in contact with partially degraded DNA molecules or with DNA at low concentrations. To mimic these natural conditions, we determined transformation efficiencies with various concentrations (0.1 ng to 30 μg) of genomic DNA extracted from strain GMI1004. Results presented in Fig. 3 indicate a relationship in the range of 1 ng to 30 μg between transformation frequency and DNA concentration. For the number of cells considered (2.5×10^7), the optimum was found to be 1 ng of DNA before the number of transformants decreased proportionally to the increase in the DNA concentration. However, a DNA amount

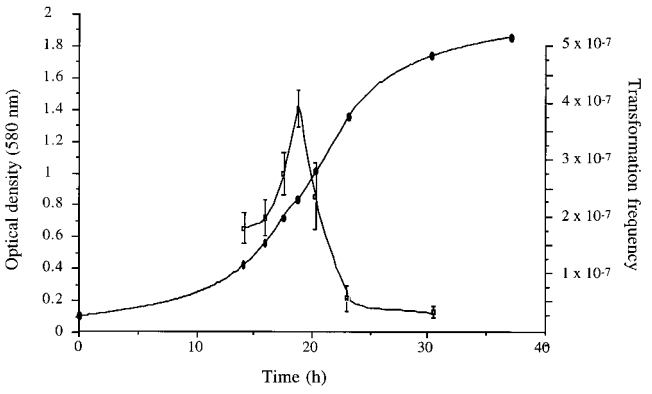

FIG. 1. Detection of a competent state during bacterial growth. Optical density measurements (●) at various times permitted the determination of growth kinetics. In parallel, bacterial cells harvested at the same times were mixed with 10 ng of DNA to determine transformation frequencies (□). Standard errors are indicated by vertical bars.

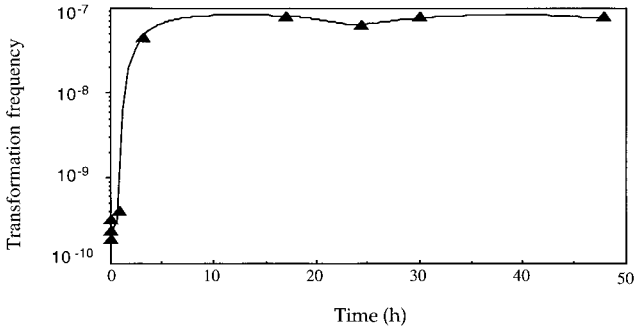

FIG. 2. Kinetics of DNA uptake by competent cells. Contact between extracellular donor DNA and competent cells was artificially interrupted by addition of DNase at different times. Conditions of DNase treatments, defined by Paget et al. (20), included the addition of 30 μl of a DNase I (type IIs; Sigma) solution (80 μg · ml^{-1}) on the membrane and incubation for 15 min at 37°C. Standard errors are not indicated, but preliminary experiments showed the same results.

of less than 1 ng resulted in a marked decrease in the transformation frequency.

On the other hand, shearing of the genomic DNA of the donor strain to provide fragments varying in size from less than 2 kb to more than 23 kb was used to simulate the presence of degraded molecules in the environment. Genomic DNA of strain GMI1004 was also sonicated in a Vibra cell cup horn (Bioblock, Illkirch, France) operating at a power setting of 30 W for 3 s. The fragments generated were separated by centrifugation through sucrose density gradients as described by Maniatis et al. (16). DNA solutions were dialyzed against Tris-EDTA buffer and standardized to a concentration of 1 μg ml^{-1} to be used as donor DNA in accordance with the transformation protocol. The mean size of the fractionated fragments harvested in each sample was evaluated by electrophoresis (2, 4, 8, 14, and 18 kb). The results (not shown) clearly demonstrate that the number of transformants decreased proportionally to the mean size of the donor DNA fragments, with a maximum decrease of 1 order of magnitude. In *P. stutzeri*, transformation frequencies decreased more than 10-fold for DNA between 10 and 1 kb (4). However, transformation in bacteria such as *B. subtilis* is sensitive to the shearing treatment since DNA fragments of 2 kb had a transformation efficiency 10,000-fold lower than that of fragments longer than 25 kb (18). For *R. solanacearum*, the decrease might be due to the sequence-specific binding of DNA to competent cell surface sites as a preliminary step for transformation (9). However, according to what is known about the dependence on substrate

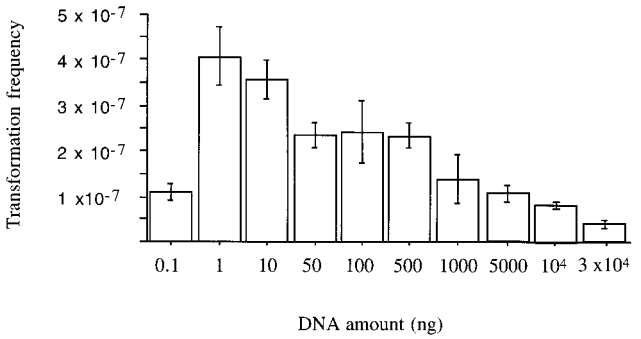

FIG. 3. Effect of donor DNA amounts on transformation efficiency. Standard errors are indicated by vertical bars.

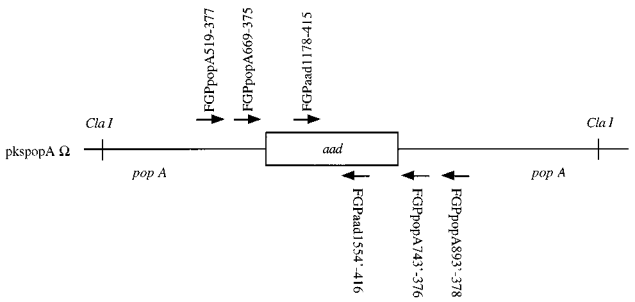

FIG. 4. DNA fragments used for the transformation of *R. solanacearum*. Plasmid pkspopAΩ was used as a template plasmid for PCR amplification and restriction digestion. Amplification with primers determined by the *popA* gene sequence was done for 35 cycles with 100 ng of pkspopAΩ, with initial denaturation for 3 min at 95°C and subsequent cycles consisting of 1 min of denaturation at 95°C, 1 min at 55°C, and 2 min at 72°C. To check the transformants, the presence of the aad^+ marker gene was detected by PCR with both primers FGPaad1172-415 and FGPaad1554′-416. In this case, the elongation time was 30 s.

length in homologous recombination in *Escherichia coli* (26), the 10-fold recombination efficiency decrease is more likely due to the small size of the fragments. Additional experiments are necessary to decide between these hypotheses.

To complete the study on the effect of transformant DNA length, we designed donor DNA in which homologous DNA regions flanking a marker gene varied in size. This was done by using fragments from recombinant plasmid pKSpopA::Ω (1) equipped with the *popA* gene (the structural gene for PopA1 proteins belonging to the *hrp* regulon) from *R. solanacearum* GMI1000 into which the Ω cassette (23) bearing the aad^+ streptomycin resistance gene had been stably inserted. Two sets of primers (FGPpopA519-377 plus FGPpopA893′-378 and FGPpopA669-375 plus FGPpopA743′-376) have been delineated to amplify the Ω cassette and increasing parts (50 and 200 bp) of the *popA* gene (Fig. 4). Moreover, digestion of the pKSpopA::Ω plasmid with the *Cla*I enzyme provided a restriction fragment harboring more-than-700-bp-long *popA* gene sequences on each side of the Ω cassette (Fig. 4). These fragments were subsequently precipitated and the pellet was dissolved in the appropriate amount of Tris-EDTA buffer to provide a 4-$\mu g \cdot ml^{-1}$ DNA concentration and used as donor DNA to transform strain GMI1000 in accordance with the standard protocol. Results clearly indicated that transformation efficiencies were directly related to the size of the homologous DNA region. Frequencies increased to 4.2×10^{-8} or 7×10^{-8}, respectively, when 200- or 719-bp-long homologous DNA regions were used (Table 2). When the bacteria were transformed with the PCR products providing 50-bp-long homologous DNA regions, transformation occurred at low but significant frequencies (5.37×10^{-9}) which were 14-fold higher than the spontaneous mutation rates. The presence of the aad^+ marker gene in the genome of the transformants was checked by PCR with primers FGPaad1172/415 and FGPaad1554′/416, which are complementary to part of the aad^+ gene (Fig. 4). All of the clones yielded a 382-bp-long DNA fragment similar in size to that obtained with plasmid pKSpopA::Ω, confirming that transformation actually occurred. For *R. solanacearum*, the minimal efficient processing fragment was less than or equal to 50 bp, although for *B. subtilis*, a 70-bp-long minimum DNA region was necessary for homologous recombination (12).

TABLE 2. Effect of the length of flanking homologous DNA regions on transformation efficiency

Sequence similarity (bp)[a]	Transformation frequency[c]
50	$5.37 (\pm 4.6) \times 10^{-9}$
200	$4.25 (\pm 0.35) \times 10^{-8}$
719, 907[b]	$7.05 (\pm 0.95) \times 10^{-8}$

[a] Length of the *popA* homologous region flanking the Ω interposon.
[b] Sequence similarity is 719 bp on one side and 907 bp on the other.
[c] The selected markers were streptomycin and spectinomycin resistances for each transformation. Standard errors from replicate experiments are in parentheses.

The hypothesis concerning the involvement of a specific DNA sequence for DNA uptake, as demonstrated with *N. gonorrhoeae* and *H. influenzae* (7), is not supported by the former experiment. Fifty base pairs of homologous DNA is sufficient for DNA uptake in the case of *R. solanacearum*, and it is very unlikely for a specific sequence to occur in such a short fragment.

DNA sequence similarity required for homologous recombination. Various studies provide evidence that transfer of chromosomal genes by transformation can occur across species and even higher taxonomic boundaries (15). The source of the donor DNA and the recipient bacteria have to be considered in evaluating the potential of transformations. This was done with *R. solanacearum* in experiments in which the donor DNA originated from a range of bacterial strains. The use of chromosomal DNA from *R. solanacearum* GMI7024, a spontaneous streptomycin-resistant mutant of strain K60, reduced the transformation efficiency more than fourfold (8.8×10^{-8}) compared to that obtained with control DNA originating from strain GMI1000 (results not shown). Moreover, when DNAs from streptomycin-resistant bacterial strains such as the *E. coli* 1832 R388 (6), *Burkholderia cepacia* ATCC 25416 (2), and *Agrobacterium tumefaciens* GMI9023 (25) were used, the transformation frequencies never diverged significantly from the spontaneous mutation rates (results not shown). This showed the inefficiency of foreign sources of DNA in transforming *R. solanacearum*, which would then belong to the group of naturally competent bacteria that accept only DNA from their own species.

The lack of transformants with foreign linear DNA is probably not due to a DNA restriction system, since successful transformants were obtained with recombinant plasmid DNA issued from *E. coli* in numerous instances (data not shown). Moreover, it has been previously demonstrated that *R. solanacearum* could be naturally transformed by broad-host-range plasmids such as RP4 and RSF1010, indicating that these foreign replicons were efficiently internalized and could autonomously replicate (13). An alternative hypothesis could be related to a decrease of the similarity of sequences between transforming and recipient DNAs, as found in *E. coli*, for which a reduction of similarity from 100 to 90% decreased the recombinant frequency over 40-fold (26). Moreover, the mismatch correction system (24), actived by DNA heteroduplex formation, could reduce gene transfer.

The homologous recombination step probably contributed by limiting transformation when the donor DNA exhibited a marked nucleotide divergence, as can be expected when it originates from remotely related organisms. *R. solanacearum* might therefore contribute to interkingdom transfer only if environmental conditions allow this bacterium to develop a competent state and if its genome possesses homologies with the transforming DNA. On the other hand, according to our findings on the stage of competence during cell growth and to those on plant colonization kinetics (10), the possibility of in planta gene exchange within the *R. solanacearum* bacterial

population itself cannot be excluded. There are some similarities in the transformation process to that of the human pathogen *N. gonorrhoeae* (28). This bacterium also exhibits a strict selection for its own DNA and develops extensively in host tissues (31). Moreover, it has been demonstrated that transformation contributes to its rapid adaptation to changing medium conditions by allowing a spread among the population of the genes involved in the survival (15). The role such a mechanism could have in the adaptation of *R. solanacearum* to soil and plant conditions, and how strongly it could be involved in plant pathogenicity, remains to be investigated.

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