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Transforming DNA Uptake Gene Orthologs Do Not Mediate Spontaneous Plasmid Transformation in *Escherichia coli*▽†

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Spontaneous plasmid transformation of *Escherichia coli* occurs on nutrient-containing agar plates. *E. coli* has also been reported to use double-stranded DNA (dsDNA) as a carbon source. The mechanism(s) of entry of exogenous dsDNA that allows plasmid establishment or the use of DNA as a nutrient remain(s) unknown. To further characterize plasmid transformation, we first documented the stimulation of transformation by agar and agarose. We provide evidence that stimulation is not due to agar contributing a supplement of Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+}. Second, we undertook to inactivate the *E. coli* orthologues of *Haemophilus influenzae* components of the transformation machine that allows the uptake of single-stranded DNA (ssDNA) from exogenous dsDNA. The putative outer membrane channel protein (HofQ), transformation pseudopilus component (PpdD), and transmembrane pore (YcaI) are not required for plasmid transformation. We conclude that plasmid DNA does not enter *E. coli* cells as ssDNA. The finding that purified plasmid monomers transform *E. coli* with single-hit kinetics supports this conclusion; it establishes that a unique monomer molecule is sufficient to give rise to a transformant, which is not consistent with the reconstitution of an intact replicon through annealing of partially overlapping complementary ssDNA, taken up from two independent monomers. We therefore propose that plasmid transformation involves internalization of intact dsDNA molecules. Our data together, with previous reports that HofQ is required for the use of dsDNA as a carbon source, suggest the existence of two routes for DNA entry, at least across the outer membrane of *E. coli*.

The spontaneous transformation of *Escherichia coli* with plasmid DNA on nutrient-containing agar plates was described in at least three independent articles (14, 23, 24). However, no attempt to characterize the mechanism of plasmid DNA uptake has been reported. Genomic analysis revealed the presence in *E. coli* of a set of genes homologous to those required for DNA uptake in naturally transformable species, including the gram-positive *Bacillus subtilis* and *Streptococcus pneumoniae* and the gram-negative *Haemophilus influenzae* and *Neisseria gonorrhoeae* (9). The machine they potentially encode would allow the uptake of single-stranded DNA (ssDNA) from an exogenous double-stranded DNA (dsDNA) substrate in *E. coli* (Fig. 1). HofQ (called ComE in reference 7) is the ortholog of the PilQ secretin of *N. gonorrhoeae*, which constitutes a transmembrane channel required for exogenous dsDNA to traverse the outer membrane (OM) and reach the so-called transformation pseudopilus (8). According to the *Bacillus subtilis* paradigm (8), assembly of the pseudopilus requires a prepilin peptidase (PppA; called PilD in reference 7), a traffic NTPase (HofB; called PilB in reference 7), and a polytopic membrane protein (HofC; called PilC in reference 7). The pseudopilus, which would include PpdD (called PilA in reference 7), provides access for dsDNA to its receptor, YbaV (called ComE1 in reference 7), through the peptidoglycan. Degradation of one strand by an unidentified nuclease (N) would allow uptake of ssDNA through YcaI (called Rec2 in reference 7), a channel in the inner membrane. Finally, DprA (also named Smf) would be required to protect internalized ssDNA from endogenous nucleases, as shown in *S. pneumoniae* (4), and to assist the processing of ssDNA into transformants (16).

In *H. influenzae*, transformation genes are preceded by unusual CRP (for cyclic AMP receptor protein) binding sites, now called CRP-S (7), that absolutely require a second protein, Sxy (also called TfoX), in addition to CRP for induction (19). Interestingly, bioinformatics analysis revealed the conservation of CRP-S sites in front of the corresponding *E. coli* genes (7), including all of the genes encoding the proteins shown in Fig. 1 (except GspD). Furthermore, some of these genes were experimentally demonstrated to require CRP, cAMP (CRP's allosteric effector), and Sxy for induction in *E. coli*, providing support to the view that CRP-S sites control a bona fide transformation regulon in this bacterium (7). However, the involvement of *E. coli* transformation genes in DNA uptake has not been documented, except for *hofQ*, which was reported to be required for the use of dsDNA as a nutrient (11, 18). Although the functionality of the *E. coli* transformation genes has not been confirmed experimentally, it is of note that the bioinformatics identification of a complete set of transformation genes in two other species not previously known to be naturally transformable, *Streptococcus thermophilus* and *Bacillus cereus*, opened the way to the demonstration of genetic transformation in these species (6, 15a).

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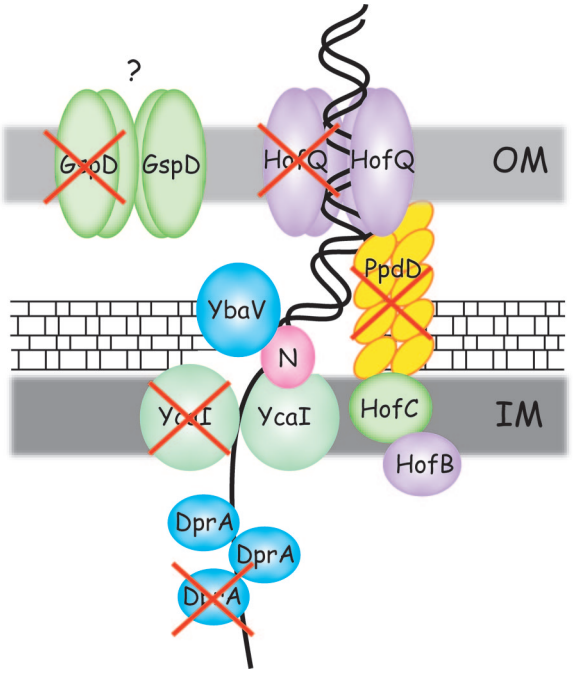

FIG. 1. Diagrammatic representation of the putative *E. coli* DNA uptake machine. The *E. coli* orthologues of proteins required involved in the uptake of transforming DNA in naturally transformable species, including *B. subtilis*, *S. pneumoniae*, *H. influenzae*, and *N. gonorrhoeae*, were identified by genomic analysis (9). GspD is a PilQ paralogue (25% identity over 278 residues), which was considered in the present study as a possible alternative route for dsDNA across the OM. A prepilin peptidase (PppA; called PilD in reference 7) required for maturation and export of proteins constituting the transformation pseudopilus (see Table S1 in the supplemental material) is not drawn on this diagram. (Additional information regarding the relationship between *E. coli* and *H. influenzae* transformation genes, and a table listing the various alternative names used in the literature are available in the supplemental material.). Red crosses indicate components of the putative DNA uptake machine inactivated during this work. IM, inner membrane.

To characterize further spontaneous plasmid transformation in *E. coli*, we first identified parameters affecting plasmid transformation frequencies on plates. We then undertook to inactivate genes encoding the putative transformation-related DNA uptake machinery of *E. coli* (Fig. 1) and to compare the rate of spontaneous plasmid transformation in the corresponding mutants and in their wild-type parent. In addition, to get an insight into the process of plasmid DNA entry, we characterized the kinetics of plasmid monomer transformation because it was shown in *S. pneumoniae* that regeneration of an intact plasmid replicon requires the independent uptake (via the transformation machine) of complementary ssDNA from two monomers (21). Finally, we discuss the possible significance of our data regarding the entry of exogenous dsDNA in *E. coli* in the light of previous findings on the use of dsDNA as a carbon source in this species (11, 18).

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers and transformation of *E. coli* on plates. All of the strains and plasmids used in the present study are listed, together with primers, in Table 1. Plasmid transformation was carried out by using a previously described procedure (23) with only minor modifications. Briefly, 5 ml of LB broth inoculated with a single colony were incubated for 14 h at 37°C with shaking. Then, 50 μl was inoculated into 5 ml of LB broth. After 14 h of incubation at 37°C, 1 ml was transferred in a beaker (4-cm diameter, 6-cm height) covered by an air-permeable membrane. After 17 h of static incubation at 37°C (~0.5-ml remaining volume, optical density at 600 nm of 4.5 to 5.0), during which the number of CFU (~2×10^9 CFU ml^{-1}) was previously reported to remain stable (23), pDsRED plasmid DNA (final concentration, 50 μg ml^{-1}) was added to culture aliquots (40 to 50 μl), which were then plated on 20-ml LB-agar plates containing ampicillin (100 μg ml^{-1}). (Note that because transformation occurs on plates [23; see also Results], the effective concentration of DNA is difficult to assess.)

To investigate the relationship between cell density and plasmid transformation efficiency, pDsRED plasmid DNA (final concentration, 50 μg ml^{-1}) was added to 50 μl of twofold serial dilutions from a ZK126 culture that had been incubated for 14 h at 37°C under static conditions in open tube, as described previously (23). Each dilution was then spread on selective plate containing 9% agar.

To purify pDsRED monomers, plasmid DNA extracted from strain ZK126 was electrophoresed on agarose gel. The band corresponding to CCC monomers was cut and DNA was recovered by using the QIAquick extraction kit (Qiagen).

Inactivation of putative DNA uptake and processing *E. coli* genes. Genes were inactivated in strain ZK126 using the one-step procedure described for inactivation of chromosomal genes with the Red recombinase (10). Primers used for inactivation of *hofQ*, *ppdD*, *ycaI*, and *dprA* (*smf*) through insertion of a chloramphenicol resistance (*cat* gene from plasmid pKD3; 1,014 bp) or a kanamycin resistance (*kan* gene from plasmid pKD4; 1,447 bp) cassette, and the mutant strains thus generated are listed in Table 1. Control PCR experiments confirmed the loss of wild-type gene fragments in the mutant strains and their replacement by a fragment, the size of which was fully consistent with that predicted from simple insertion of antibiotic-resistance gene cassette (see Fig. S1A and B and Tables S1 and S2 in the supplemental material).

RESULTS

Effect of agar concentration on plasmid transformation frequency. A possible effect of agar on transformation rate was suspected: first, because of a reduction in transformation frequencies noticed in Wuhan subsequent to a change in the agar supplier (agar powder from Wuhan Zhonghe Technology and then Bacto agar from Becton Dickinson) and, second, because of differences in transformation rates between the Wuhan and Toulouse laboratories. To examine the effect of agar concentration, wild-type *E. coli* ZK126 (K-12 strain) and RR1 (K-12×B hybrid strain) cells prepared using a previously described procedure (23) with slight modifications (see Materials and Methods) were mixed with pDsRED plasmid DNA, and 40-μl aliquots were immediately plated (~8×10^7 CFU per plate) on LB-ampicillin with agar (Kalys microbiological agar) concentrations varying between 3 and 9% (Fig. 2). A 100-fold increase in red Amp^r transformants was observed with plates containing 9% agar compared to 3%. The maximum plasmid transformation frequency as a fraction of recipient cells plated ranged between $1 \times \sim 10^{-5}$ and $\sim 4 \times 10^{-6}$ for strains RR1 and ZK126, respectively. Strain ZK126 was then used for all experiments reported hereafter to facilitate comparisons with previously published work on the use of dsDNA as a carbon source (11, 18). A similar effect of agar concentration was observed with strain ZK126 using 1 to 5% Bacto agar (Fig. 2). Spontaneous plasmid transformation was also investigated by using LB-agarose plates (Biowest Agarose; Gene Tech Company, Ltd., Shanghai, China). The transformation rates were similar to those observed on LB-agar plates, and increasing agarose concentrations also stimulated transformation (see Fig. S2A in the supplemental material).

Effect of Ca^{2+} and EGTA on rate of plasmid transformation. The positive effect of agar concentration could be chem-

TABLE 1. Strains, plasmids, and primers used in this study

E. coli strain, plasmid, or primer	Relevant genotype,[a] primer sequence,[b] and/or description	Source or reference
E. coli strains		
ZK126	W3110 Δ*lacU169 tna*-2	11
ZK126 *yhiR* mutant	*yhiR*::Tn*10*d-Cam^r	11
JPC1001	ZK126 containing pKD46 (Red recombinase plasmid)	This study
JPC1002	ZK126 *ycaI*::*cat*; Cm^r	This study
JPC1003	ZK126 *dprA*::*cat*; Cm^r	This study
JPC1004	ZK126 *hofQ*::*cat*; Cm^r	This study
JPC1005	ZK126 *gspD*::*kan*; Kan^r	This study
JPC1006	ZK126 *ppdD*::*cat*; Cm^r	This study
Plasmids		
pKD46	Red recombinase-expressing plasmid; Amp^r	10
pKD3	Chloramphenicol resistance gene, *cat*; Cm^r	10
pKD4	Kanamycin resistance gene, *kan*; Kan^r	10
pDsRED	Red fluorescence protein-expressing plasmid; Amp^r	23
Primers		
GSPD H1P1	CGTACCCCGCTTGATAAATGTTTCCGTCGGGGAACTTACAGGAATGAATGgtgtaggct ggagctgcttc; *gspD*; (−59, −10)	This study
GSPD H2P2	TACGGTGAGTGAATTCTCATATGAATGCCTCACCGTGACGATGGCGCAGGcatatgaat atcctccttag; *gspD*; (+1933, +1982)	This study
GSPD 1	GCGCTTAATGGCATTGTACTCAC; *gspD*; (−526, −504)	This study
GSPD 2	CGTTTTTCGGCGATATCAAGAC; *gspD*; (+2468, +2502)	This study
HOFQ H1P1	CGTTCCGGTAGCTCAGGTGTTGCAGGCGCTGGCTGAACAgtgtaggctggagctgcttc; *hofQ*; (+90, +128)	18
HOFQ H2P2	CGTGGCGTGATAAACACCACTAACTCGCGTCGTTCATCTcatatgaatatcctccttag; *hofQ*; (+1182, +1220)	18
HOFQ 1	TATTGCATTGTGCCTTTTAACCGG; *hofQ*; (−287, −64)	This study
HOFQ 2	AATACTCCAGCGGGTTTGGCAA; *hofQ*; (+1501, +1522)	This study
PPDD H1P1	GACAAGCAACGCGGTTTTACACTTATCGAACTGATGGTGgtgtaggctggagctgcttc; *ppdD*; (+4, +42)	This study
PPDD H2P2	GGCGTCATCAAAGCGGAAGACATCTTCGCAGGCTTGCTGcatatgaatatcctccttag; *ppdD*; (+397, +435)	This study
PPDD 1	CTTCGTAACGCCTCGCAAA; *ppdD*; (−145, −127)	This study
PPDD 2	CCGCAACATGAACCACCTC; *ppdD*; (+517, +535)	This study
SMF/DPRA H1P1	ATCACTGACCAATCGCAAAGATTGCTAAGGCTGCTTATGGCAGGGAGATAcatatgaat atcctccttag; *dprA* (*smf*); (−53, −4)	22
SMF/DPRA H2P2	GCTGCGATCCATCCTGCTAACTCCAGTTCGAGTAGTTGAGTAACTACCTCgtgtaggctg gagctgcttc; *dprA* (*smf*); (1063, 1112)	22
SMF/DPRA 1	TCTTGATCCACACGCAACTCAGCTTCTG; *dprA* (*smf*); (−185, −159)	22
SMF/DPRA 2	TTAGCAACTTTGCGAAGCCGCTCGTCC; *dprA* (*smf*); (+1140, +1167)	22
YCAI H1P1	ATGAAAATAACGACAGTCGGTGTATGCATAATTAGCGGAAgtgtaggctggagctgcttc; *ycaI*; (+1, +40)	This study
YCAI H2P2	CAGGAATGGTTATTTCCTTTATCGTTGCTACCTTGTAAAGcatatgaatatcctccttag; *ycaI*; (+1823, +1862)	This study
YCAI 1	TGCTCTAGAAAGACTTGCCACCCAGCA; *ycaI*; (−473, −452)	This study
YCAI 2	CGGGATCCCCTGAAGACGCATTCGGTT; *ycaI*; (+2302, 2323)	This study
YHIR 1	ggatccATGCTCAGTTATCGCCACAGCTTTC; *yhiR*; (+1, +25)	This study
YHIR 2	ggatccTTACTCCGGCACGATCCAGCTTACG; *yhiR*; (+819, +843)	This study

[a] Cm^r, chloramphenicol resistance; Amp^r, ampicillin resistance; Kan^r, kanamycin resistance.
[b] Lowercase letters indicate nucleotide extensions complementary to antibiotic resistance cassettes introduced in the primers to amplify either the *cat* gene (from plasmid pKD3) or the *kan* gene (from plasmid pKD4). The corresponding gene and the positions of the first and final nucleotides (in parentheses) are indicated (with respect to the ATG of the gene) following each primer sequence.

ical, owing to the presence of inducer(s) of transformation or the presence of some divalent cations in the agar preparation. Because it was reported that a Ca^{2+} concentration as low as 1 to 2 mM was sufficient to promote transformation of *E. coli* in freshwater (3), we first investigated the effect of Ca^{2+}. According to the supplier, Ca^{2+} in Bacto agar is 1,790 ppm. Agar at 5% should therefore contribute a supplement of 2.24 mM Ca^{2+}. We checked whether the addition of 2 to 4 mM Ca^{2+} to 1% agar plates would lead to plasmid transformation frequencies similar to those observed with 5% agar plates. The addition of Ca^{2+} could not restore plasmid transformation on 1% agar plates; with 4 mM Ca^{2+} added, the transformation frequency on 1% agar plates remained >800-fold lower than that on 5% agar plates (Fig. 3A). The addition of 4 mM Ca^{2+} had also no significant effect on the transformation frequency on 5% agar plates (Fig. 3A). These data ruled out the possibility that the stimulatory effect of agar on spontaneous transformation frequencies of *E. coli* on plates was due to a supplement of Ca^{2+}.

Nevertheless, the experiment in Fig. 3A did not rule out the

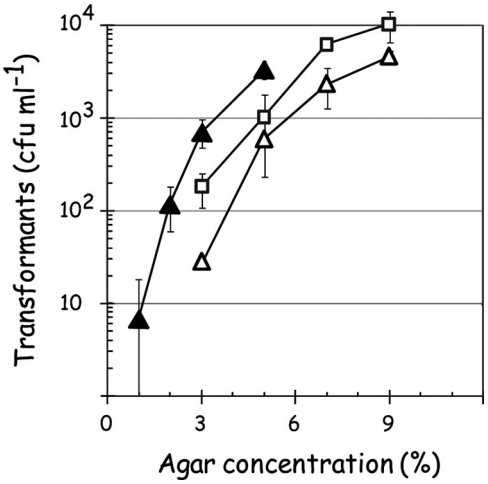

FIG. 2. Effect of agar concentration on plasmid transformation efficiency. A total of 40 μl (~8×10^7 CFU) of ZK126 (△) or RRI (□) cells that had been incubated under static conditions as described in Materials and Methods were mixed with pDsRED plasmid DNA and immediately spread onto selective plates (prepared the day before and kept with lid at 37°C) with different agar concentrations (Kalys microbiological agar). A total of 50 μl of ZK126 cells were plated with different agar concentrations (Bacto agar) (▲).

possible requirement for some cation for transformation on plates. Therefore, although it was previously reported that addition of 2 mM EGTA did not reduce plasmid transformation (23), we reinvestigated the effect of EGTA. We observed a significant inhibition of transformation (Fig. 3B). The same amount of EGTA had a smaller effect in the presence of 5% agar than 2.5% agar, which would be consistent with decreased chelation of divalent cation(s) because they are more abundant in 5% agar. An attempt was made to identify the cation(s) involved by comparing the transformation rates on 1 and 5% agar plates with or without added Fe^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+}. At the concentrations used, none of these divalent cations improved transformation (see Fig. S3 in the supplemental material).

The addition of EGTA resulted in a decrease in transformation frequencies on agarose plates stronger than that observed with agar plates (compare Fig. S2B in the supplemental material with Fig. 3B). These data would be consistent with the requirement for a cation whose concentration is lower in agarose than in agar but still sufficient to allow plasmid transformation in agarose plates.

Effect of cell density on the frequency of plasmid transformation on plates. The effect of agar or agarose concentration on transformation frequencies provided additional support to the previous conclusion (23) that plasmid transformation occurs on agar plates. This conclusion prompted us to investigate whether cell density on the plate had any effect on the rate of transformation. A transformation experiment in which the total number of CFU deposited per plate was varied between 8×10^6 and 1.28×10^8 was thus carried out. A linear relationship was observed between cell density and transformation frequency, except at low cell densities (Fig. 4). With fewer than ~2×10^7 recipient cells plated, the number of transformants observed was systematically lower than expected. This trend was observed in repeated experiments ($n > 8$) with strain ZK126, as well as with another wild-type strain (data not shown). The explanation for the apparent requirement for a minimal number of recipient cells is not known, but this observation could indicate that cell-to-cell contacts are required for plasmid transformation of *E. coli* on plates. An alternative explanation is suggested by the recent report that *Vibrio cholerae* undergoes a cell density-dependent switch from a state of extracellular DNase production in low-density populations to inhibition of DNase production by static, high-density populations in parallel with induction of transformation-dedicated genes (5). The existence of a similar release of a DNase in low-density *E. coli* populations could account for the higher than expected reduction in transformation rate when cell density is reduced.

Putative OM channel proteins and plasmid transformation. To investigate the mechanism of plasmid DNA uptake, we first inactivated the *hofQ* gene as described in Materials and Methods. Comparison of the plasmid transformation efficiency of

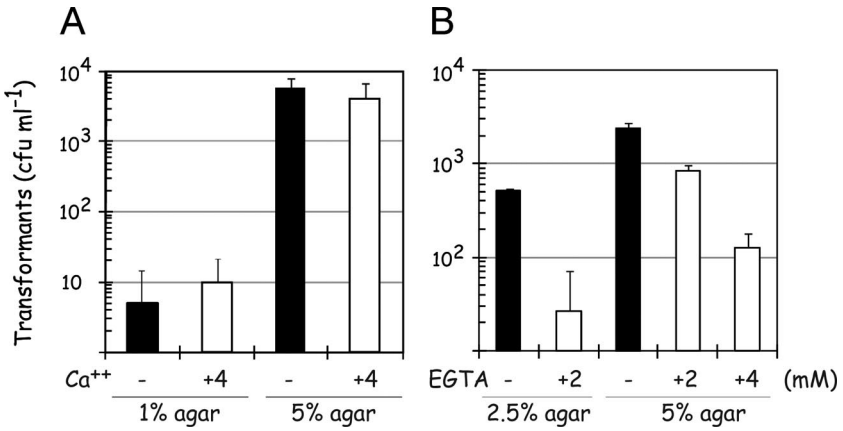

FIG. 3. Effect of Ca^{2+} (A) and EGTA (B) on plasmid transformation efficiency. Portions (50 μl) of a ZK126 culture that had been incubated under static conditions were mixed with pDsRED plasmid DNA (see Materials and Methods) and immediately spread on selective plates containing the indicated % agar (Bacto agar). (A) 0 or 4 mM Ca^{2+} was added onto the plates as indicated, respectively, by "–" and "+4". (B) EGTA at 0, 2, or 4 mM was added to the plates, as indicated, respectively, by –, +2, and +4.

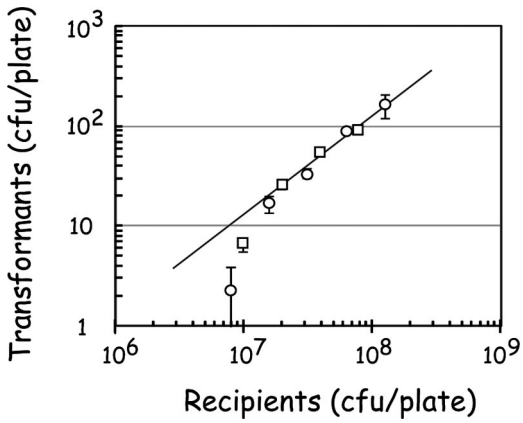

FIG. 4. Effect of cell density on plasmid transformation efficiency. Twofold serial dilutions from a ZK126 culture that had been incubated under static conditions were mixed with pDsRED plasmid DNA (see Materials and Methods), and 50 μl was immediately spread onto selective plates containing 5% agar. Squares and circles represent average values with the standard deviations from two independent experiments. The black line indicates a slope of 1.

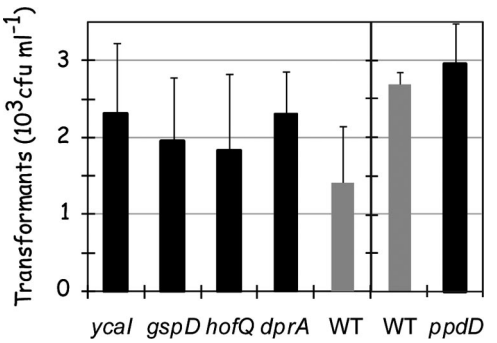

FIG. 5. *E. coli* DNA uptake gene homologs and plasmid transformation. ZK126 and its *ycaI*, *gspD*, *hofQ*, and *dprA* mutant derivatives (see Table S2 in the supplemental material) were transformed as described in the legend to Fig. 2. ZK126 and its *ppdD* mutant derivative were similarly transformed in an independent experiment.

mutant cells to that of their parental strain, ZK126, revealed no significant effect of the inactivation of *hofQ* (Fig. 5). In view of this negative result, we then considered the possible involvement of GspD, a paralogue of HofQ and of *N. gonorrhoeae* PilQ (Fig. 1), although its primary function is presumably connected to a type II protein secretion pathway (12). Inactivation of *gspD* had no detectable effect on plasmid transformation frequency (Fig. 5), ruling out the hypothesis that GspD constitutes an alternative route for entry of plasmid DNA into the periplasmic space. In addition, we observed no effect of the simultaneous inactivation of *hofQ* and *gspD* on plasmid transformation (data not shown), which excluded a possible functional redundancy of the two paralogues for the passage of double-stranded plasmid DNA across the OM.

Putative transformation-related machinery for ssDNA uptake and plasmid transformation. Inactivation of *ppdD*, *ycaI*, and *dprA* (*smf*) (see Materials and Methods) allowed us to investigate whether the putative transformation pseudopilus, the putative inner membrane channel protein for the passage of ssDNA, and the transformation-dedicated protein required for protection and processing of internalized ssDNA were required for spontaneous plasmid transformation. None of the corresponding mutant strains displayed a significant reduction in transformation frequency (Fig. 5). Finally, since *yhiR* was previously reported to be required for the use of DNA as a nutrient (11, 18), we checked the effect of its inactivation on plasmid transformation. In contrast to a previous report (23), we observed no significant difference in the transformation efficiency of *yhiR* mutant cells compared to the parental strain ZK126 (data not shown). We conclude that this gene is not required for spontaneous plasmid transformation of *E. coli* on plates.

Taken together, these data provide no support for the hypothesis that uptake of plasmid DNA proceeds in *E. coli* via the putative ssDNA uptake machinery defined on the basis of homology with components of conserved bacterial transformation machineries (Fig. 1). Since no other known candidate genes for uptake of ssDNA exist in the *E. coli* genome, we propose that plasmid DNA enters *E. coli* cells in the form of dsDNA molecules. Such a mechanism would be fully consistent with the observed dispensability of DprA (Smf), a protein normally required for protection of internalized ssDNA.

Plasmid monomers transform *E. coli* with single-hit kinetics. In *S. pneumoniae*, plasmid establishment following uptake of transforming DNA as single-stranded fragments occurs with a two-hit kinetics (21). This kinetics presumably reflects the need to associate two strands that have entered separately from two donor molecules to form a duplex that can regenerate an intact replicon. In contrast, a strong prediction of the dsDNA uptake model is that plasmid establishment in *E. coli* should require the internalization of a single dsDNA molecule per transformed cell and, therefore, should readily occur with plasmid monomers. Single-hit kinetics of transformation as a function of monomer DNA concentration are therefore expected for transformation of *E. coli* cells, as opposed to the two-hit kinetics previously observed for *S. pneumoniae* (21). To test this, pDsRED plasmid monomers were purified (see Materials and Methods), and ZK126 cells were transformed with various concentrations of monomeric DNA. Plasmid transformation showed a linear dependence on monomer concentration (Fig. 6).

The finding that monomer plasmid DNA transforms *E. coli* with single-hit kinetics is not consistent with the reconstitution of an intact replicon through annealing of partially overlapping opposite single-strands taken up from two independent monomers. We therefore propose that intact circular dsDNA molecules enter *E. coli* cells on plates to lead to plasmid establishment. Accordingly, our data suggest the existence of a route for the passage of intact double-stranded plasmid DNA through the two membranes and the peptidoglycan.

DISCUSSION

During our investigation of spontaneous plasmid transformation of *E. coli* on plates, we observed a positive impact of agar concentration on transformation frequency (Fig. 2). This observation raised the question of a possible effect of Ca^{2+} on transformation on plates. This point deserved special attention in the light of a previous publication on transformation of *E.*

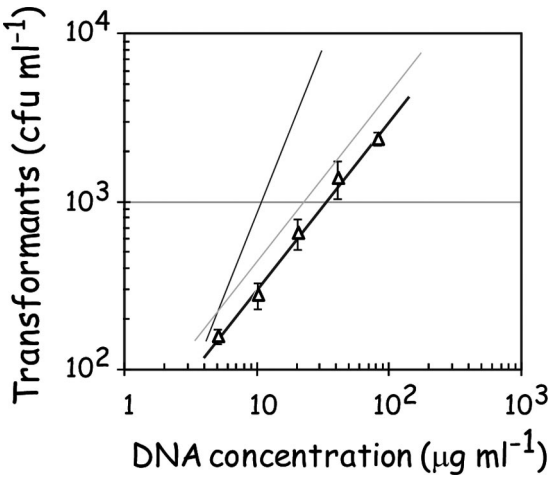

FIG. 6. Relationship between plasmid DNA concentration and transformation efficiency. Purified monomers of pDsRED plasmid DNA (final concentration ranging from 6.25 to 100 μg ml^{-1}) were added to 40-μl aliquots from a ZK126 culture, prepared as described in Materials and Methods. Each aliquot was then spread on selective plates containing 9% agar. Gray and black lines indicate slopes of 1 and 2, respectively.

coli in freshwater in which the authors reported that Ca^{2+} concentrations as low as 1 to 2 mM were sufficient to obtain transformants (3). These authors concluded that there existed a good correlation between the Ca^{2+} content of water samples and transformation frequencies. However, large variations in transformation frequencies were observed; for example, with four river water samples containing 2.0, 2.2, 2.3, and 2.4 mM Ca^{2+}, 7, 20,000, 55, and 137 transformants were, respectively, obtained per 10^8 cells (3). *E. coli* cells could even transform in the absence of Ca^{2+} but with 50 mM $MgCl_2$ (3). Our experiments establish that the stimulation of transformation by agar is unlikely to result from a supplement of Ca^{2+} (Fig. 3). This conclusion is strengthened by the finding that plasmid transformation readily occurred on agarose plates (see Fig. S2 in the supplemental material). A similar conclusion that parameters other than Ca^{2+} affect *E. coli* transformation was attained in a study of the spontaneous transformation of colonial *E. coli* on food samples, which showed that transformation occurred with frequencies unrelated to Ca^{2+} concentration (ranging from ~0.5 to ~8 mM) in the samples (14).

To account for the stimulatory effect of agar and agarose, we envision three possibilities. First, agar/agarose preparations may contain some cation(s) required for spontaneous plasmid transformation. If this is the case, then our data indicate that this cation is neither Ca^{2+} (Fig. 3) nor Fe^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} (see Fig. S3 in the supplemental material). Second, some anion(s) present in agar/agarose preparations could interfere with the surface of *E. coli* cells and change its interaction with DNA. A third, non-mutually exclusive explanation for the impact of agar/agarose concentration would be via some physical parameter. Higher agar/agarose concentrations, which are likely to result in higher meshing, could prevent the burying of DNA in the agar layer, thereby favoring contacts between *E. coli* cells and plasmid DNA on the surface of plates. This physical explanation could account for the variations in transformation rates observed with different agar sources since, according to the suppliers, the different agar preparations exhibited different gel strengths (a parameter which measures the force required to break a 1% agar gel; Wuhan Zhonghe Technology agar powder at 1,000 g/cm^2; Becton Dickinson Bacto agar at 600 g/cm^2; Kalys microbiological agar at 955 g/cm^2). However, no linear relationship between gel strength and transformation rate was apparent from our results, suggesting that if this parameter plays a role, it cannot be the only factor affecting spontaneous plasmid transformation of *E. coli* cells on plates.

Our conclusion that the stimulation of transformation by agar and agarose is not due to a supplement of Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, or Zn^{2+} does not per se rule out a possible requirement for some of these cations for transformation of *E. coli* cells on plates. The inhibitory effect of EGTA indicates that divalent cation(s) plays a role in the process. If 1% agar contains the minimal amount of any divalent cation required for full transformation proficiency, then additional amounts of this cation would not have any effect. On the other hand, chelation by EGTA of any cation that would be present in agar/agarose is expected to be more efficient with 1% agar than with 5% agar, thus accounting for the observed stronger inhibitory effect of EGTA with lower agar concentration. Alternatively, some cation other than the five assayed in the present study could be required for transformation as affinity of EGTA for polyvalent cations, such as Ca^{2+}, Mn^{2+}, Cd^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, $Fe^{2+/3+}$, and Al^{3+}, varies over a 10^7-fold range (2, 15). In any case, taking into account the large number of enzymatic activities requiring cations (e.g., Mg^{2+} for ATPases or DNases, Fe^{2+} for β-lactamases or DNA transposases, Ca^{2+} for phytase, etc.), any step in transformation, from the expression of specific genes required for DNA uptake and/or DNA processing to the functioning of proteins involved in plasmid uptake and/or establishment, could be affected through chelation by EGTA.

With regard to the mechanism of plasmid DNA uptake in *E. coli*, our data indicate that intact double-stranded plasmid DNA can traverse the OM by a mechanism that does not require HofQ. On the other hand, the fact that *hofQ* is required for the use of dsDNA as a nutrient but not for the use of nuclease-digested DNA (11) is consistent with the proposal that HofQ constitutes a channel for dsDNA to cross the OM. Taken together, these observations suggest the existence of two routes for dsDNA across the OM of *E. coli*.

Our data show that the orthologues of the *H. influenzae* OM channel (HofQ), transformation pseudopilus (PpdD), and transmembrane pore (YcaI) are not required for spontaneous plasmid transformation of *E. coli*. We conclude that plasmid DNA does not enter *E. coli* cells as ssDNA via the putative transformation machine. This conclusion is consistent with our observation that DprA is not required for plasmid transformation. This protein binds ssDNA in *B. subtilis* and *S. pneumoniae* (16) and is essential for preventing immediate degradation of internalized ssDNA in the latter species (4). On the other hand, our finding that purified plasmid monomers transform *E. coli* cells with single-hit kinetics demonstrates that a unique monomer molecule is sufficient to give rise to a transformant. Together with the observation that the putative transformation machine for ssDNA uptake is not required for plasmid trans-

formation, these data lead us to propose that spontaneous plasmid transformation of *E. coli* on plates involves entry of intact dsDNA into the cytosol.

In addition to *hofQ*, *yrfABCD*, *yhiR*, and *yhgHI* were also shown to be required for the use of dsDNA as a nutrient by *E. coli* cells (11, 18). Although these genes were identified as homologs of genes suspected or demonstrated to be required for natural transformation in the gram-negative *H. influenzae* (9, 11), their function remains elusive (see discussion in the supplemental material). In *E. coli*, it is unknown whether intact dsDNA crosses the OM and then enters the cytosol via the transformation-related ssDNA-uptake machine (Fig. 1) or whether degradation of dsDNA occurs in the periplasm and is followed by the internalization of degradation products. It would be interesting to establish whether YcaI and PpdD are required for the use of dsDNA as a carbon source. If this turned out to be the case, this would strongly suggest that, after crossing the OM through HofQ, exogenous dsDNA is converted to ssDNA and internalized as such (Fig. 1). *E. coli* cells would thus be equipped with two pathways for internalization of DNA into the cytosol, one specific for ssDNA and the other specific for dsDNA. This situation would not be unprecedented.

Two routes for DNA uptake in *Enterobacteriaceae* and *Pasteurellaceae*? The plasmid transformation of *H. influenzae* was shown to be unaffected by inactivation of *rec2*, the gene encoding the orthologue of *B. subtilis* ComEC and of *E. coli* YcaI (see Table S1 in the supplemental material), i.e., by the absence of pore for ssDNA in the inner membrane (17). In contrast, chromosomal transformation, which involves internalization of ssDNA, was abolished. This observation suggests that plasmid DNA does not enter into the cytosol as ssDNA in *rec2* mutant cells. Interestingly, transformation was favored by the closed circular form of the plasmid (17), which would be consistent with the uptake of dsDNA. In addition, inactivation of *dprA*, which affects chromosomal transformation, had no effect on plasmid transformation (13). Since *H. influenzae* DprA is likely to be involved in the protection of internalized ssDNA from endogenous nucleases as documented for *S. pneumoniae* (4), this observation provides additional support to the view that *H. influenzae* cells are also equipped with two DNA uptake systems. The first one, which is induced in cells competent for chromosomal transformation, allows the internalization of ssDNA fragments taken up from exogenous dsDNA molecules. The second one presumably allows the entry of intact circular dsDNA. As in *E. coli*, the genetic control of this second system remains unknown. It is even not known whether DNA entry relies on an active uptake system. An intriguing possibility would be the involvement in plasmid transformation of extracellular OM vesicles naturally produced by several gram-negative bacteria (10). Plasmid DNA could be first internalized by membrane vesicles as documented with *Pseudomonas aeruginosa* (20) and then be trafficked to recipient cells by the vesicles.

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