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Etude de la Transformation Plasmidique Naturelle d'Escherichia coli et de ses Relations Eventuelles avec la Compétence Programmée pour la Transformation Génétique et la Compétence dite Nutritionnelle

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ETUDE DE LA TRANSFORMATION PLASMIDIQUE NATURELLE D'*ESCHERICHIA COLI* ET DE SES RELATIONS EVENTUELLES AVEC LA COMPETENCE PROGRAMMEE POUR LA TRANSFORMATION GENETIQUE ET LA COMPETENCE DITE NUTRITIONNELLE

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Résumé

Escherichia coli n'est pas connue pour être naturellement transformable. Cependant, la première partie de mon travail de thèse contribue à démontrer que l'on peut obtenir des transformants plasmidiques chez *E. coli* sur boite Luria-Bertani agar, à partir de culture 'statiques'. J'ai montré que la concentration d'agar (ou d'agarose) dans la boîte affectait l'efficacité de transformation, mais qu'elle ne dépendait pas de la concentration de cations divalent (comme par exemple Ca^{2+}, Mn^{2+} et Mg^{2+}) qui sont normalement utilisés pour la transformation 'artificielle chimique' de cette espèce (Sun *et al.*, *FEMS Microbiol. Lett.* 2006. **265:** 249–255).

Les bactéries naturellement transformables utilisent une machinerie protéique transmembranaire, conservée entre ces espèces, pour capturer l'ADN exogène et le faire pénétrer dans les cellules. *E.coli* possède l'ensemble des gènes orthologues capables de coder pour cette machinerie. Une deuxième partie de mes travaux de thèse m'a donc amené à rechercher si la transformation par les plasmides utilisait cette machinerie. Dans ce but, j'ai inactivé les gènes clés de ce transporteur potentiel, en particulier les gènes *hofQ* et *gspD* (canal transmembranaire dans la membrane externe), et *ycaI* (canal transmembranaire dans la membrane interne). J'ai également inactivé le gène *dprA* (protection de l'ADN simple brin entrant) et le gène *sxy* qui a été récemment démontré être impliqué dans l'activation transcriptionnelle des gènes précités chez *E. coli*. Aucun de ces mutants n'est affecté pour la transformation plasmidique sur boîte. Ces résultats, complétés par l'analyse des courbes de dose-réponse de transformation en fonction de la concentration d'ADN plasmidique, suggèrent que les plasmides ne pénètrent pas sous forme simple brin, mais plutôt sous forme double brin à l'intérieur du cytoplasme de *E. coli* (Sun *et al.*, *J. Bacteriol.* 2009. **191**: 713-719).

Un troisième et dernier volet de mon travail de thèse a consisté à tenter de mieux caractériser un phénomène appelé 'compétence nutritionnelle de *E. coli*, appellation qui désigne la capacité de croître en présence d'ADN exogène comme seule source de carbone. Bien qu'il ait été proposé que cette capacité repose sur l'internalisation d'ADN via la machinerie de transformation, seul l'implication éventuelle du gène *hofQ* avait été étudiée. Pour pouvoir étudier l'effet d'autres mutants que j'avais construits, j'ai cherché à reproduire les expériences publiées de croissance de la souche ZK126 sur milieu minimum M63 contenant de l'ADN. En dépit de nombreuses tentatives et contrôles, je n'ai pas réussi à observer de croissance d'*E. coli* dans ces conditions. Ces observations négatives m'ont amené à clore mon mémoire de thèse par une discussion critique des données publiées relatives à la compéténce nutritionnelle de *E. coli*.

Summary

Naturally transformable bacteria are able to take up exogenous DNA. DNA uptake is mediated by a conserved multiprotein machinery. So far, *Escherichia coli* is not considered to belong to naturally transformable species. However, in the first part of my thesis, I established a transformation system allowing spontaneous plasmid transformation of *E. coli* cells on Luria-Bertani agar plates (Sun *et al.*, *FEMS Microbiol. Lett.* 2006. **265:** 249–255). Increasing agar or agarose concentration strongly stimulated plasmid transformation, a stimulation which was not caused by divalent cations (*e.g.* Ca^{2+}, Mn^{2+} and Mg^{2+}) that normally induce chemically-induced 'artificial transformation' of *E. coli* (Sun *et al.*, *J. Bacteriol.* 2009. **191**: 713-719).

Then, the finding that *E. coli* genome was found to contain all gene orthologues encoding the transforming DNA uptake machinery prompted me to investigate whether this machinery was required for plasmid transformation of *E. coli* on plates. I inactivated several of these genes, including *hofQ* and *gspD*, which encode putative outer membrane channel proteins; *ycaI*, which encodes the putative inner membrane channel protein; *dprA*, which encodes a protein required to protect internalized single-stranded DNA (ssDNA); and the putative central regulator of competence, *sxy*. None of these genes appeared required for spontaneous plasmid transformation on LB-agar plates, suggesting that plasmid DNA does not enter as ssDNA via the transforming DNA uptake machinery. Analysis of dose-response curves of transformation frequency as a function of DNA concentration led us to propose that plasmid DNA instead enters the cytoplasm as double stranded material (Sun *et al.*, *J. Bacteriol.* 2009. Ibid.).

The third and last part of my thesis is dedicated to a reinvestigation of so-called 'nutritional competence' of *E. coli*. Previously published work reported that *E. coli* cells are able to use DNA as the sole carbon source. While this phenomenon was proposed to rely on the above-mentioned transforming DNA uptake machinery, only the requirement for *hofQ* for nutritional competence had been checked. As I wished to investigate the possible requirement for YcaI for nutritional DNA to traverse the inner membrane of *E. coli* and, more generally, whether transcription of the DNA uptake machinery required the Sxy regulator, using the corresponding mutants I had constructed, I tried to grow *E. coli* cells on M63 minimal medium with DNA as the sole source of carbon using the published strain (i.e. ZK126) and conditions. Despite numerous attempts and controls, I could not observer any growth of *E. coli* cells under these conditions. This failure to reproduce published observations led me to conclude my thesis by an in-depth discussion of the three articles dedicated to so-called nutritional competence of *E. coli*.

A. Introduction

INTRODUCTION

In our environment, exogenous DNA can be taken up into bacterial cells and stably inherited, a natural process termed "natural transformation". The acquisition of exogenous DNA often brings bacteria new genetic traits, like drug resistance and virulence, allowing them to better survive in harsh conditions (Claverys *et al.*, 2006; Elwell and Shipley, 1980; Foster, 1983; Nikaido, 2009). Many bacterial species widely distributed have been found to be naturally transformable (Lorenz and Wackernagel, 1994). During natural transformation, the outside DNA is taken up into cells by a large multiprotein polar complex which allows a single stranded DNA (ssDNA) from the donor double stranded DNA (dsDNA) to enter into the cytoplasm (Chen and Dubnau, 2004; Claverys *et al.*, 2009; Dubnau, 1999). By contrast, *Escherichia coli* has long been thought not to be naturally transformable (Solomon and Grossman, 1996), although artificial transformation of *E. coli* has been widely used. A set of DNA uptake gene homologs has been identified in *E. coli* (Claverys and Martin, 2003). Meanwhile, several reports documented that *E. coli* was transformable under certain natural conditions (Baur *et al.*, 1996; Maeda *et al.*, 2004; Tsen *et al.*, 2002). But the association between DNA uptake gene homologs and transformation in these conditions is still not known. Interestingly, some DNA uptake gene homologs have been shown to be required for the use of exogenous DNA as a carbon source in *E. coli* (Finkel and Kolter, 2001; Palchevskiy and Finkel, 2006); the homolog of competence regulator Sxy, which controls natural transformation in *Haemophilus influenzae*, seems to regulate DNA uptake gene transcription and transformation, as well as the long-term stationary-phase competition presumably for DNA as a nutrient in *E. coli* (Sinha *et al.*, 2009). But it is neither known how DNA enters into the cytoplasm for providing the nutrient nor any roles of Sxy in regulating natural transformation or the use of nutritional DNA in *E. coli*. In this thesis, I will describe my investigation of natural plasmid transformation in *E. coli*, potential roles of homologs of DNA uptake gene and *sxy* in plasmid transformation, and so-called nutritional competence, an ability to transport exogenous DNA to the catabolism cycle. Beforehand, I will introduce our current knowledge about natural competence for transformation and 'nutritional competence', as well as the recent advance in natural competence and transformation in *E. coli*.

1. DNA uptake in natural bacterial transformation

Competence for natural bacterial transformation is a genetically controlled physiological state in which exogenous DNA is taken up into bacterial cells (Dubnau, 1999). Natural transformation is widespread in bacteria. Among taxonomic groups in bacteria, more than 70 species distributed in different taxonomic groups are found to be naturally transformable (Johnsborg *et al.*, 2007; Lorenz and Wackernagel, 1994). These include *Neisseria gonorrhoeae*, *H. influenzae* and *Vibrio cholerae* belonging to proteobacteria β and γ, and Firmicutes *Bacillus subtilis* and *Streptococcus pneumoniae* (Fig. 1).

In most naturally transformable bacteria, e.g. *S. pneumoniae*, competence is transient (Claverys *et al.*, 2009); while in some rare cases, e.g. in *N. gonorrhoeae*, competence establishes all over the bacterial life cycle (Biswas *et al.*, 1989). Natural transformation is different from artificial transformation which requires heat shock/Ca^{2+} stimulation, electroporation or protoplast preparation (Dubnau, 1999). In the following paragraphs, only natural transformation will be described.
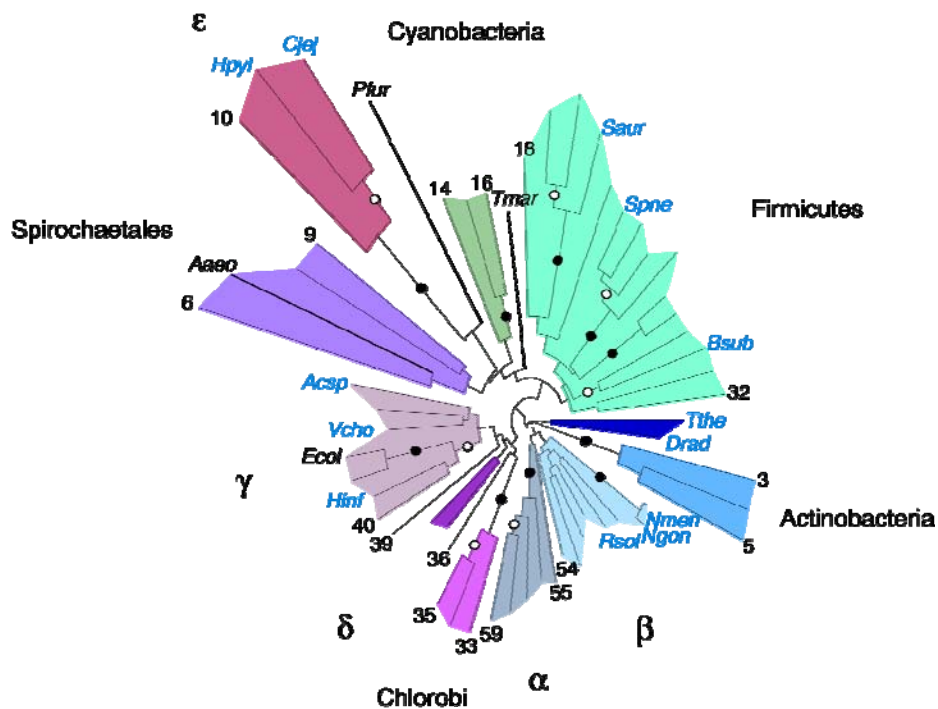
Figure 1. Distribution of naturally transformable bacteria. (reproduced from (Mortier-Barrière *et al.*, 2007)). The phylogenetic tree is based on the DprA (Smf) domain (pfam02481) detected in completely sequenced genomes from representatives of the main taxons. Bacterial species with names in blue colour are naturally transformable: *Helicobacter pylori* (*Hpyl*), *Campylobacter jejuni* (*Cjej*), *Streptococcus aureus* (*Saur*), *Streptococcus pneumoniae* (*Spne*), *Bacillus subtilis* (*Bsub*), *Thermus thermophilus* (*Tthe*), *Deinococcus radiodurans* (*Drad*), *Neisseria gonorrhoeae* (*Ngon*), *Neisseriae meningitis* (*Nmen*), *Ralstonia solancearum* (*Rsol*), .*Haemophilus influenzae* (*Hinf*), *Vibrio cholerae* (*Vcho*) and *Acinetobacter* sp. (*Acsp*).

To cope with stressful conditions, a specific bacterial species has evolved its own way to control the development of competence for acquiring exogenous DNA (Claverys *et al.*, 2006; Dubnau, 1991; Meibom *et al.*, 2005; Redfield *et al.*, 2005). For example, competence establishes to allow transformation proceeds when G+ bacterium *S. pneumoniae* is treated with antibiotics (e.g. streptomycin) (Prudhomme *et al.*, 2006), while chitin induces competence in G- bacterium *V. cholerae* (Meibom *et al.*, 2005). As the stressful conditions encountered by different bacterial species vary largely, competence regulation mechanisms are diverse among them. However, during natural transformation, these apparently different competence regulation circuits control the same set of DNA uptake gene homologs, which are thought to constitute an evolutionarily conserved DNA transport machinery (Fig. 2); as well as DNA processing gene homologs, which integrate incoming DNA in most, if not all, naturally transformable bacteria (Claverys *et al.*, 2006; Meibom *et al.*, 2005; Redfield *et al.*, 2005). In the following paragraphs, I will exemplify how bacteria sense different conditions and use disparate competence regulation circuits to control DNA uptake during natural transformation.

1.1 Competence regulation in natural transformation

Competence in the Gram-positive (G+) B. subtilis *and* S. pneumoniae.

When *B. subtilis* starts entering into the stationary phase or is shifted to a nutrient poor condition, it begins to develop competence for transformation which stands for a while (Dubnau, 1991). By contrast, *S. pneumoniae* establishes a transient competence at the exponential growth stage (Claverys *et al.*, 2009). Competence regulation in the two G+ bacteria *B. subtilis* and *S. pneumoniae* share some similarities. The transcription of genes encoding the main competence regulators ComX in *S. pneumoniae* and ComK in *B. subtilis* is stimulated by small secreted peptides, called competence-stimulating peptides (CSPs) through dedicated two-component regulatory systems (TCSs) (Claverys *et al.*, 2006). In *S. pneumoniae*, CSP is sensed by a membrane histidine kinase (HK) ComD and the interaction between CSP and ComD presumably leads to ComD self-phosphorylation (Havarstein *et al.*, 1996; Pestova *et al.*, 1996). It is assumed that ComD~P then transmits the phosphoryl group to ComE (Pestova *et al.*, 1996) and ComE~P activates the transcription of *comX* (Luo *et al.*, 2003), which encodes the main regulator of competence in *S. pneumoniae* (Lee and Morrison, 1999). Similarly, *B. subtilis* CSP (ComX) in the milieu stimulates the HK ComP, which phosphorylates its cognate response regulator ComA to activate the transcription of ComS (Dubnau, 1991). ComS prevents proteolysis of ComK, a transcription regulator stimulating

the expression of competence genes and thus ComK accumulates (Turgay *et al.*, 1998). Accumulated ComX and ComK stimulate the transcription of genes for DNA uptake and processing in *S. pneumoniae* and *B. subtilis* respectively.

The two competence regulatory cascades in *S. pneumoniae* and *B. subtilis* show evolutionary diversity. Transcription of competence genes relies on the alternative sigma factor ComX in *S. pneumoniae*, whereas a transcriptional regulator ComK is involved in *B. subtilis* (Claverys *et al.*, 2006). Although the competence signal is transmitted by TCS in both *S. pneumoniae* and *B. subtilis*, components of the TCS are unrelated (Claverys *et al.*, 2006). Additionally, the pre-CSP is processed to mature CSP by cutting of a leader peptide before being transported out of the *S. pneumiae* cell, whereas the competence pheromone ComX in *B. subtilis* is post-translationally modified in addition to cleavage of the inactive precursor (Claverys *et al.*, 2006).

Competence in the Gram-negative (G-) H. influenzae.

In G- bacterium *H. influenzae*, the transcription regulator Sxy and the cAMP receptor (CRP) collaborate in regulating the transcription of late competence genes (Cameron and Redfield, 2006; Redfield *et al.*, 2005). A special CRP binding site with the core sequence TGCGA locates in the promoters of most competence regulons in *H. influenzae* (Redfield *et al.*, 2005). This site has also been identified in promoters of competence gene homologs in other γ-proteobacteria including *E. coli* (Cameron and Redfield, 2006). In the presence of Sxy, CRP binds this special CRP binding site (called CRP-S) and initiates the transcription of these competence genes for fulfilling DNA uptake and processing tasks in later stages (Redfield *et al.*, 2005).

The case in *V. cholerae* exemplifies how bacteria evolve competence regulation mechanisms compatible with its inhabitancy. *V. cholerae* frequently lives on shellfish which have an external envelope made of chitin. The condition for inducing competence in *V. cholerae* has not been known for a long time. Recent studies showed that its envelope component chitin stimulated competence by activating the transcription regulator Sxy (Meibom *et al.*, 2005). Then activated Sxy switched on the conserved DNA uptake machinery to acquire extracellular DNA (Meibom *et al.*, 2005).

1.2 DNA transport in natural bacterial transformation

In natural transformation of both G+ and G- bacteria, expressed competence regulators start the transcription of late competence genes, which perform functions in DNA binding, uptake and processing for transporting DNA across outer membrane (OM, only for G-

bacteria) and inner membrane (IM) and integrating DNA as part of their own genetic material in the cytosol.

DNA binding and uptake through the OM
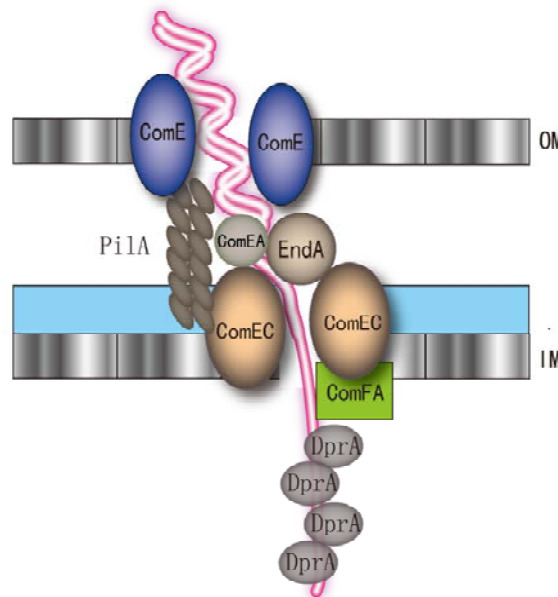
Figure 2. Components of the DNA uptake machinery in bacteria. Components of the type IV pilus (T4P) fiber, e.g. PilA in the G- bacterium *H. influenzae* and ComGC, GD, GG and GE in the G+ bacterium *B. sutilis*, are required at an early stage in DNA uptake (Chung *et al.*, 1998; Dubnau, 1997; Tracy *et al.*, 2008). A prerequisite in G- bacteria is DNA transport across the OM, which is achieved in *H. influenzae* through the porin ComE (Dougherty and Smith, 1999). It is believed that T4P allow access of exogenous dsDNA to its receptor ComEA, which then presents dsDNA to a nuclease EndA in *S. pneumonia*e. EndA degrades one strand of ssDNA with 5'→ 3' polarity; while the complementary ssDNA is transported to an IM channel formed by ComEC with 3'→ 5' polarity (Bergé *et al.*, 2002; Méjean and Claverys, 1988, 1993). The ATP-driven helicase ComFA provides energy for DNA translocation from the periplasm into the cytosol (Londono-Vallejo and Dubnau, 1994; Takeno *et al.*, 2011). Then, DprA protects the incoming ssDNA from degradation by nucleases (Bergé *et al.*, 2003) and favors the loading of the recombinase RecA, which then allows the search for homology (Mortier-Barrière *et al.*, 2007).

The first step for DNA uptake is recognizing and binding exogenous DNA. In *H. influenzae*, DNA tagged with a 9 bp special motif (5'-AAGTGCGGT-3′), called uptake signal sequence (USS), can be selectively taken up into the periplasm (Danner *et al.*, 1980; Danner *et al.*, 1982). The biased DNA uptake is predicted to be resulted from a receptor located at the OM which recognizes USS. Exogenous DNA is thought to enter into the periplasm through an OM protein, called ComE (or PilQ in *N. gonorrhoeae*) (Dougherty and Smith, 1999; Drake and Koomey, 1995; Tomb *et al.*, 1991) (Fig. 2). The diameter of the central cavity of PilQ is ~6 nm (Collins *et al.*, 2001; Parge *et al.*, 1995), enough for accommodating DNA double helix (2.4 nm). A group of proteins named pilins/pseudopilins participate in both assembly/disassembly of Type IV Pilus (T4P) and DNA uptake (Chen and Dubnau, 2004)

(Fig. 2). T4P allows DNA to access its membrane-associated receptor ComEA for further transport which will be discussed in the following section (Fig. 2).

DNA translocation through the IM

Next, DNA is translocated from the periplasm to the cytoplasm. The incoming DNA contacts with its membrane-attached receptor ComEA, which presents dsDNA to EndA for degrading one strand of ssDNA so as to allow the other complementary ssDNA to be transported through IM channel consisting of ComEC/Rec2, a multispanning membrane protein with seven transmembrane segments (Barouki and Smith, 1985; Bergé *et al.*, 2002; Draskovic and Dubnau, 2005; Inamine and Dubnau, 1995) (Fig. 2). In the best documented transformation model as concerns uptake polarity and kinetics, *S. pneumoniae*, it was shown that during the translocation of DNA, one strand of ssDNA from the dsDNA enters into the cytoplasm in a 3'→ 5' direction while the complement ssDNA is degraded in a reverse direction (Bergé *et al.*, 2002; Méjean and Claverys, 1988, 1993) (Fig. 2). Degradation of the complementary ssDNA is dependent on a nuclease EndA (Bergé *et al.*, 2002), first identified in *Diplococcus* (*Streptococcus*) *pneumoniae* as required for DNA uptake (Lacks *et al.*, 1975; Lacks and Neuberger, 1975). As the rates for ssDNA entry and its complement degradation are approximately identical and they proceed simultaneously, the two events are thought to be coupled (Bergé *et al.*, 2002; Méjean and Claverys, 1988, 1993; Puyet *et al.*, 1990). The channel for DNA translocation across IM is supposed to be constituted by two ComEC monomers (Draskovic and Dubnau, 2005). In an experiment for re-examining the function of Rec-2 in *H. influenzae*, radiolabelled donor DNA can be recovered from the periplasm of a *rec-2* mutant but not from the periplasm of the wild type; by contrast, the radioactive labels can be detected in the genomic DNA of the wild type but not of the *rec-2* mutant (Barouki and Smith, 1985). In a systematic analysis of DNA uptake in *S. pneumoniae*, DNA binding was not significantly impaired in a *comEC* mutant while DNA entry was almost eliminated in that mutant (Bergé *et al.*, 2002). The experimental evidence, together with bioinformatic analysis, strongly suggest that ComEC serves as a gate keeper for exogenous DNA passing across IM to enter into the cytoplasm. However, direct evidence supporting the above hypothesis is still missing. Because over-expression of ComEC/Rec-2 homologs (including YcaI in *E. coli*) is toxic in *E. coli* (Daley *et al.*, 2005; Inamine and Dubnau, 1995), it is not accessible to analyze a wild type ComEC/Rec-2 protein by biochemical means. With the assistance from an ATP-binding protein ComFA, ssDNA is transported into the cytosol through an IM channel (Londono-Vallejo and Dubnau, 1994; Takeno *et al.*, 2011) (Fig. 2). The translocated ssDNA is

extremely sensitive to nucleases in the cytoplasm. In *S. pneumoniae*, it was shown that internalized DNA is immediately degraded when DprA or RecA is missing (Mortier-Barrière *et al.*, 2007).

1.3 DNA uptake for repair, recombination (genetic plasticity) or nutrition?

The conservation of DNA uptake machine in far-related bacteria causes people to think about its evolutional role. Three non-mutual exclusive hypotheses, DNA for repair, DNA for recombination and DNA for food, have been proposed to explain the evolutionary function of DNA uptake (Chen and Dubnau, 2004; Dubnau, 1999). The DNA for repair hypothesis proposes that internalized DNA could serve as templates for restoring the damaged chromosomal region (Bernstein *et al.*, 1985). This theory is supported by the findings that DNA repair machinery is induced as part of the competence regulon and that transformation increased survival in UV-irradiated populations in *B. subtilis* (Haijema *et al.*, 1996; Hoelzer and Michod, 1991; Love *et al.*, 1985; Michod *et al.*, 1988; Wojciechowski *et al.*, 1989). However, both of the two lines of evidence were cast into doubt later (Dubnau, 1999). First, the induction of DNA repair machinery as part of the competence regulon is not a strong prediction on the DNA uptake for repair theory, as expressed DNA repair proteins could provide preventive protection to avoid further damage on DNA (Dubnau, 1999). Second, with either a cloned fragment or total chromosomal DNA, the effect of transformation on cell survival is similar in *H. influenzae* (Dubnau, 1999). These indicate that the damaged DNA should not be the target for the incoming donor DNA (Dubnau, 1999).

The DNA for recombination proposal is straightforward and reasonable (Claverys *et al.*, 2009; Dubnau, 1999). Under natural conditions, bacteria in diverse niches establish competence to meet their special survival requirements through disparate strategies. These different competence induction circuits convergently regulate the same DNA uptake machinery to acquire new genes, which often bring bacteria new genetic traits to help increase their fitness (Claverys *et al.*, 2009; Dubnau, 1999).

It has been proposed that the main purpose of DNA uptake is providing nutrients (Redfield *et al.*, 1997). In *H. influenzae*, competence is induced by transferring cells to a nutrient poor medium. The transcription of competence genes are induced by the increase of cyclic AMP, a small molecule which is produced when energy sources are limited (Dorocicz *et al.*, 1993; Macfadyen *et al.*, 1996). Moreover, the presence of purine nucleotides or nucleosides in the nutrient poor medium prevents the transcription of the competence genes

and transformation (MacFadyen *et al.*, 2001). The regulation of competence in *H. influenzae* seems fit the prediction of the nutrient hypothesis (Redfield *et al.*, 1997). Dubnau pointed out that it would be wasteful to use DNA as a nutrient with the DNA uptake machine because only one strand of ssDNA was assimilated and the other strand was discarded (Dubnau, 1999). Claverys *et al.* argue that DprA is induced as a competence gene and this protein protects the incoming ssDNA from degradation; "maintenance of *dprA* in the minimal com regulon of *H. influenzae* is suggestive of a strong selection pressure to keep internalized ssDNA intact, a feature of no use for catabolism" (Claverys *et al.*, 2006).

1.4 Impact of DNA uptake mechanism on plasmid transformation in naturally transformable species

In *S. pneumoniae*, it was shown that internalized DNA is immediately degraded when DprA or RecA is missing (Bergé *et al.*, 2003; Mortier-Barrière *et al.*, 2007). For plasmid transformation, monomeric plasmid transforms *S. pneumoniae* with a two-hit kinetics pattern (Saunders and Guild, 1981). This reveals that two plasmid molecules are required to generate a transformant, implying that the (re)generation of a double-stranded plasmid DNA requires the annealing in the cytoplasm of two complementary ssDNA strands taken up from different donor plasmid molecules (Saunders and Guild, 1981).

2. Natural competence and transformation in *E. coli*?

2.1 An *E. coli* machinery for DNA uptake conserved from naturally transformable species?

It has not been thought that *E. coli* is naturally transformable for a long time (Solomon and Grossman, 1996). However, by comparing with the model bacteria *B. subtilis* and *H. influenzae* for studying natural transformation, a complete set of DNA uptake gene homologs and key DNA processing gene homologs are characterized in the genome of *E. coli* (Table 1). These genes encode putative DNA uptake proteins to form a membrane protein complex for the passage of DNA across OM and IM, and putative DNA processing proteins for internalizing incoming DNA in the cytoplasm (Fig. 3). Therefore, *E. coli* is potentially able to assimilate exogenous DNA through this set of DNA uptake and processing machinery but the conditions required for this machinery to be assembled remain unknown. Once this machinery

is assembled, if exogenous dsDNA is available, it might be pulled into the cytoplasm with the assistance from the putative pseudopilus fiber assembled by the pseudopilin PpdD (Fig. 3). The putative OM protein HofQ and/or GspD may serve as a conduit for the pseudopilus fiber extrusion and retraction across the OM, accompanied by the entry of extracellular DNA into the periplasm (Fig. 3). Incoming dsDNA in the periplasm may attach to the putative DNA receptor YbaV, followed by the degradation of one strand of ssDNA with 5'-end by a nuclease and the entry of the other strand of ssDNA with 3'-end across an IM channel formed by membrane protein YcaI (Fig. 3). The entered ssDNA would be bound by DprA to prevent from nuclease degradation in the cytoplasm (Fig. 3).
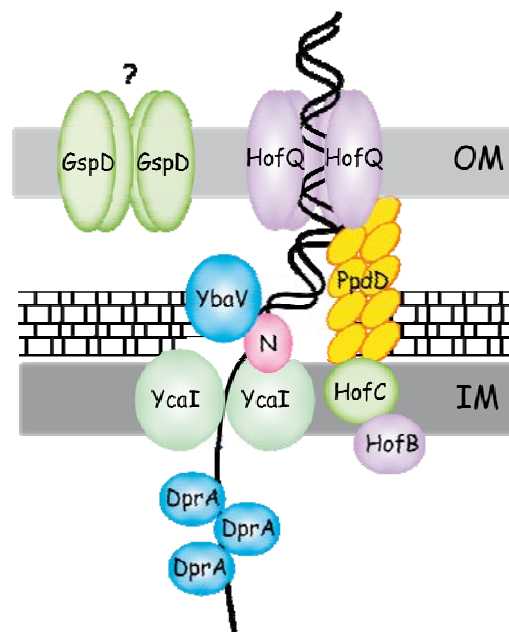
Figure 3. Putative DNA uptake machinery in *E. coli.* The competent cell is equipped with a group of DNA uptake proteins for the movement of exogenous DNA. After the contact between exogenous DNA and the competent cell, dsDNA is pulled into the cell. With the assistance from the traffic NTPase HofB and the polytopic membrane protein HofC, the pseudopilus, which is mainly consisted of the pseudopilin PpdD, mediates DNA transport across OM. The channel for passage of DNA through the OM is constituted by the porin HofQ (or its paralog GspD). In the periplasm, dsDNA contacts the integral membrane protein YbaV which mediates DNA to a nuclease (N) for degrading one strand of ssDNA and allows the other strand ssDNA to traverse through the IM channel formed by YcaI. During DNA translocation from the periplasm to the cytoplasm, degradation of one strand of ssDNA proceeds in a 5'→ 3' direction, and its complement passes through IM channel in an opposite direction. The incoming ssDNA is subsequently protected by DprA, an ssDNA protector for further processing.

2.2 A competence regulon in *E. coli*?

Sxy and CRP regulate competence for natural transformation by controlling the transcription of competence genes in *H. influenzae* (Cameron and Redfield, 2006; Redfield *et al.*, 2005). Quantitative PCR data demonstrate that *E. coli* Sxy and CRP can also regulate the

TABLE 1 *E. coli* orthologs of proteins involved in DNA uptake and processing, as well as competence regulation in *H. influenzae* and *B. subtilis*.

Protein	*E. coli*	other name	ref.	*H. influenzae*	other name	other name	other name	ref.	*B. subtilis*	ref.
The outer membrane channel										
Secretin	HofQ[a]		(5)	ComE[b]	HI0435		(PilQ)[@]	(4, 13)	n. a.	
The competence pseudopilus										
Pilins or pseudopilins	PpdD[c]		(3, 10, 16)	PilA[d]	HI0299			(4, 14)	ComGC[e]	(2)
									ComGD[e]	(2)
									ComGE[e]	(2)
									ComGG[e]	(2)
?	HofM[a]	YrfD	(3, 5, 8, 10)		HI0439	ComA[b]	(PilM)[@]	(5, 8, 9, 15)		
	HofN[a]	YrfC	(3, 5, 8, 10)		HI0438	ComB[b]	(PilN)[@]	(5, 8, 9, 15)		
	HofO[a]	YrfB	(3, 5, 8, 10)		HI0437	ComC[b]	(PilO)[@]	(5, 8, 9, 15)		
	HofP[a]	YrfA	(3, 5, 8, 10)		HI0436	ComD[b]	(PilP)[@]	(5, 8, 9, 15)		
?	GntX	YhgH	(3, 5, 8, 10)		HI0434	ComF[b]	Com101A	(5, 8, 9, 15)	ComFC[f, #]	(2)
	GntY	YghI[*]	(3, 5, 8, 10)		HI0433[*]	ComG[*]		(5, 8, 9, 15)	n. a.	
Prepilin peptidase	PppA		(3, 5, 8, 10)	PilD[d]	HI0296			(3, 9, 15)	ComC	(2)
Traffic NTPase	HofB[c]		(3, 5, 8, 10)	PilB[d,]	HI0298			(3, 9, 15)	ComGA[e]	(2)
Polytopic membrane protein	HofC[c]		(3, 5, 8, 10)	PilC[d]	HI0297			(3, 9, 15)	ComGB[e]	(2)
Pilus retraction ATpase	YggR		(11)	n. a.			(PilT)[@]	(11)	n. a.	
DNA translocation machinery										
DNA receptor	YbaV		(3)	ComE1	HI1008			(3, 9)	ComEA[g]	(2)
(Inner) membrane channel	YcaI		(3)	Rec2	HI0061			(1, 3, 7)	ComEC[g]	(2)
DNA translocase	n. i.			n. i.					ComFA[f]	(2)
DNA processing										
ssDNA binding and RecA loading	DprA/Smf		(12)	DprA				(6)	Smf	(2)
Competence regulation										
Competence-specific regulator	Sxy[§]		(11)	Sxy				(9)	n. a.	
Transcription regulator	CRP[§]		(11)	CRP				(9)	n. a.	
	n. a.			n. a.					ComK	(2)

Names in red correspond to proteins with a demonstrated role in DNA uptake or transformation (*B. subtilis* and *H. influenzae*) or in 'nutritional' DNA uptake (*E. coli*) or in regulating competence; Names in green correspond to proteins encoded in operons in which at least one gene is required for transformation; **Names in black** correspond to proteins for which no data exist to indicate involvement in transformation; n. a., not applicable; n. i., not identified; [a] *hofMNOPQ* operon; [b] *comABCDEF* operon; [c] *ppdD-hofBC* operon; [d] *pilABCD* operon; [e] *comG* [*ABCDEG*] operon; [f] *comF* [*ABC*] operon; [g] *comE* [*AC*] operon; [@] Name of *N. gonorrhoeae* homologs; [#] Inactivation of *comFC* was reported to reduce transformation by 5 to 10-fold, to have no effect on DNA binding and only a limited effect on DNA uptake. The exact role of ComFC thus remains unknown; [*] Although *yhgHI* (*gntY*) was described as a homolog of a gene shown to play a role in *H. influenzae* (*comG*), no data exist to indicate involvement in competence and transformation of this gene (also called *orfG*) in this species;

§ Although the role of Sxy and CRP in 'nutritional competence' remains unknown, they were reported to regulate the expression of *hofQ* and *ppdD* in *E. coli*.

References for Table 1

1   **Barouki, R., and H. O. Smith.** 1985. Reexamination of phenotypic defects in *rec-1* and *rec-2* mutants of *Haemophilus influenzae* Rd. J Bacteriol **163:**629-34.

2   **Chen, I., and D. Dubnau.** 2004. DNA uptake during bacterial transformation. Nat Rev Microbiol **2:**241-9.

3   **Claverys, J. P., and B. Martin.** 2003. Bacterial "competence" genes: signatures of active transformation, or only remnants? Trends Microbiol **11:**161-5.

4   **Dougherty, B. A., and H. O. Smith.** 1999. Identification of *Haemophilus influenzae* Rd transformation genes using cassette mutagenesis. Microbiology **145:**401-9.

5   **Finkel, S. E., and R. Kolter.** 2001. DNA as a nutrient: novel role for bacterial competence gene homologs. J Bacteriol **183:**6288-93.

6   **Karudapuram, S., X. Zhao, and G. J. Barcak.** 1995. DNA sequence and characterization of *Haemophilus influenzae dprA+*, a gene required for chromosomal but not plasmid DNA transformation. J Bacteriol **177:**3235-40.

7   **Notani, N. K., J. K. Setlow, V. R. Joshi, and D. P. Allison.** 1972. Molecular basis for the transformation defects in mutants of *Haemophilus influenzae*. J Bacteriol **110:**1171-80.

8   **Palchevskiy, V., and S. E. Finkel.** 2006. *Escherichia coli* competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient. J Bacteriol **188:**3902-10.

9   **Redfield, R. J., A. D. Cameron, Q. Qian, J. Hinds, T. R. Ali, J. S. Kroll, and P. R. Langford.** 2005. A novel CRP-dependent regulon controls expression of competence genes in *Haemophilus influenzae*. J Mol Biol **347:**735-47.

10   **Sauvonnet, N., P. Gounon, and A. P. Pugsley.** 2000. PpdD type IV pilin of *Escherichia coli* K-12 can be assembled into pili in *Pseudomonas aeruginosa*. J Bacteriol **182:**848-54.

11   **Sinha, S., A. D. Cameron, and R. J. Redfield.** 2009. Sxy induces a CRP-S regulon in *Escherichia coli*. J Bacteriol **191:**5180-95.

12   **Smeets, L. C., S. C. Becker, G. J. Barcak, C. M. Vandenbroucke-Grauls, W. Bitter, and N. Goosen.** 2006. Functional characterization of the competence protein DprA/Smf in *Escherichia coli*. FEMS Microbiol Lett **263:**223-8.

13   **Tomb, J. F., H. el-Hajj, and H. O. Smith.** 1991. Nucleotide sequence of a cluster of genes involved in the transformation of *Haemophilus influenzae* Rd. Gene **104:**1-10.

14   **Tracy, E., F. Ye, B. D. Baker, and R. S. Munson, Jr.** 2008. Construction of non-polar mutants in *Haemophilus influenzae* using FLP recombinase technology. BMC Mol Biol **9:**101.

15   **VanWagoner, T. M., P. W. Whitby, D. J. Morton, T. W. Seale, and T. L. Stull.** 2004. Characterization of three new competence-regulated operons in *Haemophilus influenzae*. J Bacteriol **186:**6409-21.

16   **Yoshida, N., T. Nakajima-Kambe, K. Matsuki, and T. Shigeno.** 2007. Novel plasmid transformation method mediated by chrysotile, sliding friction, and elastic body exposure. Anal Chem Insights **2:**9-15.

transcription of putative competence genes by binding with the CRP-S site in their promoters (Sinha *et al.*, 2009). Over-expressing *sxy* increased the transcription of 34 CRP-S transcriptional units (TUs), 14 TUs of the σ^{32} regulon and 13 TUs of the flagellar-chemotaxis regulon(Sinha *et al.*, 2009). Among the 34 CRP-S TUs, 7 of them contain putative competence genes *hofMNOPQ* (*yrfABCD* and *hofQ*), *ppdD-hofBC*, *yggR*, *ybaV*, *ycaI*, *dprA* and *gntX* listed in Table 1 (Sinha *et al.*, 2009). The transcription of all these potential DNA uptake/processing genes listed above are decreased in the absence of cAMP (Sinha *et al.*, 2009), indicating the involvement of cAMP receptor CRP in the transcriptional regulation of competence genes. Increased transcription of *ppdD* due to ectopic expression of Sxy enabled the synthesis of the pilin encoded by *ppdD* in the presence of CRP, although type IV pili were not detected (Sinha *et al.*, 2009), supporting the view that Sxy controls the synthesis of competence proteins at transcriptional level.

2.3 So-called nutritional competence of *E. coli*

Although *E. coli* competence gene homologs have not been assigned any roles in genetic transformation, one of them, *hofQ*, whose homolog encodes a putative OM porin for DNA uptake in *H. influenzae* and *N. gonorrhoeae* (Collins *et al.*, 2001; Dougherty and Smith, 1999; Drake and Koomey, 1995; Parge *et al.*, 1995; Tomb *et al.*, 1991), is required to the use of exogenous DNA as a nutrient in *E. coli* (Finkel and Kolter, 2001; Palchevskiy and Finkel, 2006). Besides *hofQ*, seven other genes *yrfABCD*, *yhiR*, and *yhgHI* in putative competence regulons are required to the use of DNA as a nutrient as well (Palchevskiy and Finkel, 2006). 'Nutritional competence' is therefore defined to describe the ability of bacterial cells to use exogenous dsDNA as a nutrient (Palchevskiy and Finkel, 2006).

The involvement of the genes in 'nutritional competence' is determined by the following two criteria. First, when wild-type cells and mutant cells are incubated separately, both are able to grow well over a long time incubation; while when they are co-incubated in the same culture over several days, mutant cells can not compete with wild-type cells for survival and show stationary-phase-specific competition-defective. Second, the mutant can not grow in the chemical defined culture with DNA as the sole carbon source (Finkel and Kolter, 2001; Palchevskiy and Finkel, 2006). According to the above criteria, three additional genes *xonA*, *recJ* and *xseA*, which encode ssDNA exonucleases I, VII and X respectively but are not competence gene homologs, have been assigned roles in digesting incoming ssDNA for providing metabolizable carbon source (Palchevskiy and Finkel, 2009). Interestingly, when

the *sxy* mutant and its wild-type parent were cultured separately in LB broth, both showed a similar growth pattern. However, when the two strains were co-incubated in the same medium for a long time, the *sxy* mutant died more rapidly than the wild type, a phenotype that was tentatively attributed to the inability of *sxy* cells to compete for the use of DNA as food (Sinha *et al.*, 2009). However, there is not direct evidence supporting the association between *sxy* and the use of DNA as the sole carbon source in minimal medium.

2.4 'Natural' plasmid transformation in *E. coli*

Compared to the relatively well defined 'nutritional competence', little is known about natural competence for plasmid transformation in *E. coli*. In the widely used chemical transformation, it requires a high concentration of divalent cations (100 mM Ca^{2+}, 50 mM Mg^{2+} or Mn^{2+}), low temperature incubation and temperature shift are crucial (Cohen *et al.*, 1972; Hanahan, 1983; Mandel and Higa, 1970). Recent studies revealed that plasmid transformation of *E .coli* occurred under certain natural conditions (Baur *et al.*, 1996). By incubating cells in water samples from mineral springs, Baur *et al*. showed that *E. coli* was transformable in water containing Ca^{2+} as low as 2 mM (Baur *et al.*, 1996). They observed that, either incubating cells in 10 mM $CaCl_2$ without temperature shift or incubating cells with a lower $CaCl_2$ concentration but with a temperature shift, could establish a moderate competence in *E. coli* (Baur *et al.*, 1996). Therefore, *E. coli* is transformable in the absence of one or several factors for inducing artificial transformation. Inactivation of the putative competence regulator encoding *sxy* did not affect either electroporation or chemical transformation induced by $RbCl_2$ and heat shock, but did decrease plasmid transformation frequency from 9.1×10^{-8} to less than 2.3×10^{-9} in 1 mM $CaCl_2$ (Sinha *et al.*, 2009). This result suggests that Sxy may regulate plasmid transformation at a low Ca^{2+} concentration.

3 Thesis objectives

3.1 Pending questions regarding natural plasmid transformation and 'nutritional competence' in *E. coli*.

The identification of competence gene homologs through *E. coli* genome analysis and evidence that Sxy controls a competence regulon suggest that *E. coli* cells (sections 2.1 and 2.2) might develop competence spontaneously. However, conditions leading to induction of

the putative Sxy-dependent regulon remain unknown. On the other hand, the ability of *E. coli* cells to use DNA as a nutrient was interpreted as a reflection of their ability to develop competence (section 2.3); and observations on natural plasmid transformation in *E. coli* (section 2.4) could also result from uptake of plasmid DNA by the transformation machinery.

However, a number of key observations are missing to definitely connect 'nutritional competence' and/or plasmid transformation to natural transformation mediated by the Sxy-controlled regulon.

- First, the genetic requirements for the uptake of DNA during plasmid transformation have not been investigated.

- Second, as concerns nutritional competence, while it has been shown that HofQ is required for the use of DNA as a nutrient (section 2.3), it remains unknown whether ssDNA translocates through the IM via the putative transmembrane channel YcaI (Figure 3).

- Third, the possible dependence on Sxy of plasmid transformation and of nutritionlal competence has not been investigated.

These are the main questions I tried to answer during my thesis.

3.2 Experimental approaches and presentation of data

During this Thesis, I first defined conditions for natural plasmid transformation of *E. coli* cells on plates. This published work constitutes Chapter 1 (*Escherichia coli* is naturally transformable in a novel transformation system. *FEMS Microbiol. Lett.* 2006. **265:** 249–255).

I then investigated whether transforming DNA uptake gene orthologs are required for plasmid transformation under the conditions I set up. This published work is presented as Chapter 2 (Transforming DNA Uptake Gene Orthologs Do Not Mediate Spontaneous Plasmid Transformation in *Escherichia coli*. *J. Bacteriol.* 2009. **191**: 713-719). As an addendum, during a visit in the laboraoty of Rosie Redfield, I checked whether the competence regulator gene homolog *sxy* is required for plasmid transformation of *E. coli* cells on plates. The corresponding experiments are presented as Supplemental Material to Chapter 2.

Chapter 3 summarizes my attempts at further investigating so-called nutritional competence of *E. coli*. It is followed by a Supplemental Discussion of previous reports regarding the use of DNA as a nutrient and its association with competence gene homologs in *E. coli*.

Finally, these three 'Result' Chapters are followed by a General Discussion/Conclusions on both natural plasmid transformation and 'nutritional competence' of *E. coli*.

References

Barouki, R., and Smith, H.O. (1985) Reexamination of phenotypic defects in *rec-1* and *rec-2* mutants of *Haemophilus influenzae* Rd. *J Bacteriol* **163**: 629-634.

Baur, B., Hanselmann, K., Schlimme, W., and Jenni, B. (1996) Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. *Appl Environ Microbiol* **62**: 3673-3678.

Bergé, M., Moscoso, M., Prudhomme, M., Martin, B., and Claverys, J.P. (2002) Uptake of transforming DNA in Gram-positive bacteria: a view from *Streptococcus pneumoniae*. *Mol Microbiol* **45**: 411-421.

Bergé, M., Mortier-Barriere, I., Martin, B., and Claverys, J.P. (2003) Transformation of *Streptococcus pneumoniae* relies on DprA- and RecA-dependent protection of incoming DNA single strands. *Mol Microbiol* **50**: 527-536.

Bernstein, H., Byerly, H.C., Hopf, F.A., and Michod, R.E. (1985) The evolutionary role of recombinational repair and sex. *Int Rev Cytol* **96**: 1-28.

Biswas, G.D., Thompson, S.A., and Sparling, P.F. (1989) Gene transfer in *Neisseria gonorrhoeae*. *Clin Microbiol Rev* **2 Suppl**: S24-28.

Cameron, A.D., and Redfield, R.J. (2006) Non-canonical CRP sites control competence regulons in *Escherichia coli* and many other gamma-proteobacteria. *Nucleic Acids Res* **34**: 6001-6014.

Chen, I., and Dubnau, D. (2004) DNA uptake during bacterial transformation. *Nat Rev Microbiol* **2**: 241-249.

Chung, Y.S., Breidt, F., and Dubnau, D. (1998) Cell surface localization and processing of the ComG proteins, required for DNA binding during transformation of *Bacillus subtilis*. *Mol Microbiol* **29**: 905-913.

Claverys, J.P., and Martin, B. (2003) Bacterial "competence" genes: signatures of active transformation, or only remnants? *Trends Microbiol* **11**: 161-165.

Claverys, J.P., Prudhomme, M., and Martin, B. (2006) Induction of competence regulons as a general response to stress in gram-positive bacteria. *Annu Rev Microbiol* **60**: 451-475.

Claverys, J.P., Martin, B., and Polard, P. (2009) The genetic transformation machinery: composition, localization, and mechanism. *FEMS Microbiol Rev* **33**: 643-656.

Cohen, S.N., Chang, A.C., and Hsu, L. (1972) Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. *Proc Natl Acad Sci U S A* **69**: 2110-2114.

Collins, R.F., Davidsen, L., Derrick, J.P., Ford, R.C., and Tonjum, T. (2001) Analysis of the PilQ secretin from *Neisseria meningitidis* by transmission electron microscopy reveals a dodecameric quaternary structure. *J Bacteriol* **183**: 3825-3832.

Daley, D.O., Rapp, M., Granseth, E., Melen, K., Drew, D., and von Heijne, G. (2005) Global topology analysis of the *Escherichia coli* inner membrane proteome. *Science* **308**: 1321-1323.

Danner, D.B., Deich, R.A., Sisco, K.L., and Smith, H.O. (1980) An eleven-base-pair sequence determines the specificity of DNA uptake in *Haemophilus* transformation. *Gene* **11**: 311-318.

Danner, D.B., Smith, H.O., and Narang, S.A. (1982) Construction of DNA recognition sites active in *Haemophilus* transformation. *Proc Natl Acad Sci U S A* **79**: 2393-2397.

Dorocicz, I.R., Williams, P.M., and Redfield, R.J. (1993) The *Haemophilus influenzae* adenylate cyclase gene: cloning, sequence, and essential role in competence. *J Bacteriol* **175**: 7142-7149.

Dougherty, B.A., and Smith, H.O. (1999) Identification of *Haemophilus influenzae* Rd transformation genes using cassette mutagenesis. *Microbiology* **145**: 401-409.

Drake, S.L., and Koomey, M. (1995) The product of the *pilQ* gene is essential for the biogenesis of type IV pili in *Neisseria gonorrhoeae*. *Mol Microbiol* **18**: 975-986.

Draskovic, I., and Dubnau, D. (2005) Biogenesis of a putative channel protein, ComEC, required for DNA uptake: membrane topology, oligomerization and formation of disulphide bonds. *Mol Microbiol* **55**: 881-896.

Dubnau, D. (1991) Genetic competence in *Bacillus subtilis*. *Microbiol Rev* **55**: 395-424.

Dubnau, D. (1997) Binding and transport of transforming DNA by *Bacillus subtilis*: the role of type-IV pilin-like proteins--a review. *Gene* **192**: 191-198.

Dubnau, D. (1999) DNA uptake in bacteria. *Annu Rev Microbiol* **53**: 217-244.

Elwell, L.P., and Shipley, P.L. (1980) Plasmid-mediated factors associated with virulence of bacteria to animals. *Annu Rev Microbiol* **34**: 465-496.

Finkel, S.E., and Kolter, R. (2001) DNA as a nutrient: novel role for bacterial competence gene homologs. *J Bacteriol* **183**: 6288-6293.

Foster, T.J. (1983) Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. *Microbiol Rev* **47**: 361-409.

Haijema, B.J., van Sinderen, D., Winterling, K., Kooistra, J., Venema, G., and Hamoen, L.W. (1996) Regulated expression of the *dinR* and *recA* genes during competence development and SOS induction in *Bacillus subtilis*. *Mol Microbiol* **22**: 75-85.

Hanahan, D. (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557-580.

Havarstein, L.S., Gaustad, P., Nes, I.F., and Morrison, D.A. (1996) Identification of the streptococcal competence-pheromone receptor. *Mol Microbiol* **21**: 863-869.

Hoelzer, M.A., and Michod, R.E. (1991) DNA repair and the evolution of transformation in *Bacillus subtilis*. III. Sex with damaged DNA. *Genetics* **128**: 215-223.

Inamine, G.S., and Dubnau, D. (1995) ComEA, a *Bacillus subtilis* integral membrane protein required for genetic transformation, is needed for both DNA binding and transport. *J Bacteriol* **177**: 3045-3051.

Johnsborg, O., Eldholm, V., and Havarstein, L.S. (2007) Natural genetic transformation: prevalence, mechanisms and function. *Res Microbiol* **158**: 767-778.

Lacks, S., Greenberg, B., and Neuberger, M. (1975) Identification of a deoxyribonuclease implicated in genetic transformation of *Diplococcus pneumoniae*. *J Bacteriol* **123**: 222-232.

Lacks, S., and Neuberger, M. (1975) Membrane location of a deoxyribonuclease implicated in the genetic transformation of *Diplococcus pneumoniae*. *J Bacteriol* **124**: 1321-1329.

Lee, M.S., and Morrison, D.A. (1999) Identification of a new regulator in Streptococcus pneumoniae linking quorum sensing to competence for genetic transformation. *J Bacteriol* **181**: 5004-5016.

Londono-Vallejo, J.A., and Dubnau, D. (1994) Mutation of the putative nucleotide binding site of the *Bacillus subtilis* membrane protein ComFA abolishes the uptake of DNA during transformation. *J Bacteriol* **176**: 4642-4645.

Lorenz, M.G., and Wackernagel, W. (1994) Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol Rev* **58**: 563-602.

Love, P.E., Lyle, M.J., and Yasbin, R.E. (1985) DNA-damage-inducible (*din*) loci are transcriptionally activated in competent *Bacillus subtilis*. *Proc Natl Acad Sci U S A* **82**: 6201-6205.

Luo, P., Li, H., and Morrison, D.A. (2003) ComX is a unique link between multiple quorum sensing outputs and competence in *Streptococcus pneumoniae*. *Mol Microbiol* **50**: 623-633.

Méjean, V., and Claverys, J.P. (1988) Polarity of DNA entry in transformation of *Streptococcus pneumoniae*. *Mol Gen Genet* **213**: 444-448.

Méjean, V., and Claverys, J.P. (1993) DNA processing during entry in transformation of *Streptococcus pneumoniae*. *J Biol Chem* **268**: 5594-5599.

Macfadyen, L.P., Dorocicz, I.R., Reizer, J., Saier, M.H., Jr., and Redfield, R.J. (1996) Regulation of competence development and sugar utilization in *Haemophilus influenzae* Rd by a phosphoenolpyruvate:fructose phosphotransferase system. *Mol Microbiol* **21**: 941-952.

MacFadyen, L.P., Chen, D., Vo, H.C., Liao, D., Sinotte, R., and Redfield, R.J. (2001) Competence development by *Haemophilus influenzae* is regulated by the availability of nucleic acid precursors. *Mol Microbiol* **40**: 700-707.

Maeda, S., Sawamura, A., and Matsuda, A. (2004) Transformation of colonial *Escherichia coli* on solid media. *FEMS Microbiol Lett* **236**: 61-64.

Mandel, M., and Higa, A. (1970) Calcium-dependent bacteriophage DNA infection. *J Mol Biol* **53**: 159-162.

Meibom, K.L., Blokesch, M., Dolganov, N.A., Wu, C.Y., and Schoolnik, G.K. (2005) Chitin induces natural competence in *Vibrio cholerae*. *Science* **310**: 1824-1827.

Michod, R.E., Wojciechowski, M.F., and Hoelzer, M.A. (1988) DNA repair and the evolution of transformation in the bacterium *Bacillus subtilis*. *Genetics* **118**: 31-39.

Mortier-Barrière, I., Velten, M., Dupaigne, P., Mirouze, N., Pietrement, O., McGovern, S., Fichant, G., Martin, B., Noirot, P., Le Cam, E., Polard, P., and Claverys, J.P. (2007) A key presynaptic role in transformation for a widespread bacterial protein: DprA conveys incoming ssDNA to RecA. *Cell* **130**: 824-836.

Nikaido, H. (2009) Multidrug resistance in bacteria. *Annu Rev Biochem* **78**: 119-146.

Palchevskiy, V., and Finkel, S.E. (2006) *Escherichia coli* competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient. *J Bacteriol* **188**: 3902-3910.

Palchevskiy, V., and Finkel, S.E. (2009) A role for single-stranded exonucleases in the use of DNA as a nutrient. *J Bacteriol* **191**: 3712-3716.

Parge, H.E., Forest, K.T., Hickey, M.J., Christensen, D.A., Getzoff, E.D., and Tainer, J.A. (1995) Structure of the fibre-forming protein pilin at 2.6 A resolution. *Nature* **378**: 32-38.

Pestova, E.V., Havarstein, L.S., and Morrison, D.A. (1996) Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. *Mol Microbiol* **21**: 853-862.

Prudhomme, M., Attaiech, L., Sanchez, G., Martin, B., and Claverys, J.P. (2006) Antibiotic stress induces genetic transformability in the human pathogen *Streptococcus pneumoniae*. *Science* **313**: 89-92.

Puyet, A., Greenberg, B., and Lacks, S.A. (1990) Genetic and structural characterization of endA. A membrane-bound nuclease required for transformation of *Streptococcus pneumoniae*. *J Mol Biol* **213**: 727-738.

Redfield, R.J., Schrag, M.R., and Dean, A.M. (1997) The evolution of bacterial transformation: sex with poor relations. *Genetics* **146**: 27-38.

Redfield, R.J., Cameron, A.D., Qian, Q., Hinds, J., Ali, T.R., Kroll, J.S., and Langford, P.R. (2005) A novel CRP-dependent regulon controls expression of competence genes in *Haemophilus influenzae*. *J Mol Biol* **347**: 735-747.

Saunders, C.W., and Guild, W.R. (1981) Monomer plasmid DNA transforms *Streptococcus pneumoniae*. *Mol Gen Genet* **181**: 57-62.

Sinha, S., Cameron, A.D., and Redfield, R.J. (2009) Sxy induces a CRP-S regulon in *Escherichia coli*. *J Bacteriol* **191**: 5180-5195.

Solomon, J.M., and Grossman, A.D. (1996) Who's competent and when: regulation of natural genetic competence in bacteria. *Trends Genet* **12**: 150-155.

Takeno, M., Taguchi, H., and Akamatsu, T. (2011) Role of ComFA in controlling the DNA uptake rate during transformation of competent *Bacillus subtilis*. *J Biosci Bioeng*.

Tomb, J.F., el-Hajj, H., and Smith, H.O. (1991) Nucleotide sequence of a cluster of genes involved in the transformation of *Haemophilus influenzae* Rd. *Gene* **104**: 1-10.

Tracy, E., Ye, F., Baker, B.D., and Munson, R.S., Jr. (2008) Construction of non-polar mutants in *Haemophilus influenzae* using FLP recombinase technology. *BMC Mol Biol* **9**: 101.

Tsen, S.D., Fang, S.S., Chen, M.J., Chien, J.Y., Lee, C.C., and Tsen, D.H. (2002) Natural plasmid transformation in *Escherichia coli*. *J Biomed Sci* **9**: 246-252.

Turgay, K., Hahn, J., Burghoorn, J., and Dubnau, D. (1998) Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *Embo J* **17**: 6730-6738.

Wojciechowski, M.F., Hoelzer, M.A., and Michod, R.E. (1989) DNA repair and the evolution of transformation in *Bacillus subtilis*. II. Role of inducible repair. *Genetics* **121**: 411-422.

B. Results

Chapter 1

Escherichia coli is Naturally Transformable in a Novel Transformation System

This work was published (**Sun, *et al.*** 2006. *Escherichia coli* is naturally transformable in a novel transformation system. FEMS Microbiol Lett **265:**249-55) and is reproduced hereafter.

The reprint is followed by a Technical Annex containing a Figure summarizing the new transformation protocol and a Table summarizing the suggestions for improvement of spontaneous plasmid transformation of *E. coli* cells on solid medium.

Escherichia coli is naturally transformable in a novel transformation system

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Abstract

A novel transformation system, in which neither a nonphysiological concentration of Ca^{2+} and temperature shifts nor electronic shocks were required, was developed to determine whether *Escherichia coli* is naturally transformable. In the new protocol, *E. coli* was cultured normally to the stationary phase and then cultured statically at 37 °C in Luria–Bertani broth. After static culture, transformation occurred in bacteria spread on Luria–Bertani plates. The protein synthesis inhibitor chloramphenicol inhibited this transformation process. The need for protein synthesis in plated bacteria suggests that the transformation of *E. coli* in this new system is regulated physiologically.

Introduction

More than 40 bacterial species are known to be naturally transformable (Lorenz & Wackernagel, 1994). *Escherichia coli* is not considered to be naturally transformable (Solomon & Grossman, 1996), although various artificial transformation methods (in which Ca^{2+}, incubation at low temperature and a temperature shift or an electronic shock is necessary) have been developed and are widely used in molecular biology (Mandel & Higa, 1970; Hanahan, 1983). Recently, observations of transformation at low Ca^{2+} concentrations and of the physiological changes accompanying the development of competence suggest that *E. coli* is likely to be naturally transformable (Huang & Reusch, 1995; Baur *et al.*, 1996; Xie *et al.*, 2000; Li *et al.*, 2001; Tsen *et al.*, 2002; Maeda *et al.*, 2004, 2006).

However, in these studies, low-temperature treatments and/or temperature shifts were unavoidable. So the natural transformability of *E. coli* is still in question because of the lack of knowledge about the conditions favourable for transformation, which might differ from those used in standard artificial transformation. Does current knowledge of natural transformation in *E. coli* reflect its natural transformability or is this a misconception because the conditions for inducing natural competence are unknown? *E. coli* has genes homologous to those involved in natural genetic transformation in *Haemophilus influenzae* and *Neisseria gonorrhoeae*, and in *E. coli* these genes are involved in the utilization of DNA as a nutrient source (Finkel & Kolter, 2001; Palchevskiy & Finkel, 2006). This suggests that *E. coli* itself may be naturally transformable, but it was not possible to test whether these genes were actually involved in the transformation process in *E. coli* (Claverys & Martin, 2003) as the appropriate conditions for natural transformation were not known. However, at the gene level, these clues support the idea that *E. coli* is naturally transformable.

To test whether *E. coli* is naturally transformable, we designed a novel transformation system that is completely different from the traditional artificial transformation method. In the new transformation system, addition of Ca^{2+}, low-temperature treatment and a temperature shift were not required. Here, we describe the natural transformation of *E. coli* with the new protocol.

Materials and methods

Strains, media and plasmids

The *E. coli* strains and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown in Luria–Bertani (LB) medium. Plasmid pUC18-cm was derived from pUC18 by inserting the *cat* gene from pTnMod-Ocm (Dennies & Zylstra, 1998) into the *Sac*I site. Plasmid pDsRED (Tolker-Nielsen *et al.*, 2000), derived from

Table 1. Strains and plasmids used

Strain or plasmid	Description	Source or reference
Strains		
HB101	F^- supE44 hsdS20 (r_B^- m_B^-) recA 13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 metl-1	Laboratory reserve
JM109	F' traD36 lacI^{q}Δ(lacZ)M15 pro A+B+/e14$^-$ (McrA –)Δ(lac-proAB)thi gyrA96 (Nal^r)endA1 hsdR17(r_k^- m_k^+)relA1 supE44 recA1	CCTCC*
TG1	F' traD36 proA$^+$B$^+$ lacI^{q}Δ(lacZ) M15/supEΔ(lac-proAB) hsdRΔ5 thi	Laboratory reserve
DH5α	F^- supE44ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1	CCTCC
BL21 (DE3)	F^- ompT hsdS$_B$ (r_B^- m_B^-) gal dcm (DE3)	Novagen
Plasmids		
pTnMod-Ocm	TnMod variant containing a cat gene flanked by two Sac I sites	Dennies & Zylstra (1998)
pUC18-cm	pUC18 carrying the cat gene, Cam^r, Ap^r	This study
pDsRED	pUC18 carrying the red fluorescence gene, Ap^r	Tolker-Nielsen et al. (2000)
pSU-A	p15A replicon, Ap^r	Unpublished data

*CCTCC: China Center for Type Culture Collection.

pUC19, contains a gene that encodes a red fluorescent protein and confers ampicillin resistance. Plasmids pDsRED, pUC18-cm and pSU-A (unpublished data) were isolated using a U-gene Miniprep kit (Anhui U-gene Biotechnology Co., Ltd) as specified by the manufacturer. DNA preparations were resuspended in sterile distilled water and vortexed gently before use. The concentrations of plasmids pDsRED, pUC18-cm and pSU-A solutions were determined by their absorbance at 260 nm.

Transformation protocol

All experiments were performed at 37 °C. *Escherichia coli* strains were grown overnight in LB broth (to reach a density of $10^8 \sim 10^9$ CFU mL^{-1}). The culture was inoculated at 1 : 100 (v/v) into 5 mL of fresh LB broth and then cultured with shaking. When culture growth reached the stationary phase, cells were transferred to microfuge tubes. Fifty microlitres of the culture ($10^7 \sim 10^8$ CFU) was inoculated into each tube and the tube was left open. To prevent contamination, the open tubes were placed in a sterile beaker covered with a hydrophobic fluoropore membrane. We called this an 'open system'. A closed tube was named a 'closed system' here accordingly. After the cultures had been incubated for 12 h at 37 °C without shaking in open systems or closed systems when necessary, 2 μg of plasmid was added to each tube ($10^7 \sim 10^8$ CFU). The tube contents were gently mixed and then spread on LB-agar plates supplemented with ampicillin (100 μg mL^{-1}).

To investigate the influence of static culture on transformation efficiency (Fig. 2), bacteria at different stages of culture were transferred to open systems. The cultures in open systems were incubated with or without shaking for 12 h before the addition of pDsRED and subsequent plating.

To determine the optimal time for static culture (Fig. 3), after 12 h of culture with shaking, cells were transferred to open systems and were cultured statically. At intervals,

plasmid pDsRED was added to the cultures and then gently mixed before plating.

To determine where transformation occurred, after 12 h of static culture, cells were spread on LB-agar plates containing ampicillin (100 μg mL^{-1}) immediately before pDsRED was spread on these plates.

To evaluate the effect of a calcium chelator on transformation, static cultures mixed with pDsRED were plated on LB-agar plates supplemented with ampicillin (100 μg mL^{-1}) and EGTA (2 mM).

Transformants grown on selective plates were counted about 16 h after plating.

Data analysis

The transformation efficiency and transformation frequency were calculated as the number of antibiotic-resistant CFU per μg plasmid and the number of antibiotic-resistant CFU mL^{-1}/total CFU mL^{-1}. All experiments were performed with duplicate or triplicate samples and repeated three or more times, depending on the degree of variability. The data presented are from representative experiments, and error estimates are calculated from duplicate or triplicate samples.

DNase assay

Each static culture was supplemented with 2 μg of plasmid DNA and further incubated for 1, 2, 4 or 8 h. The mixture was incubated with DNase I (500 μg mL^{-1}) for 20 min before plating (Wang *et al.*, 2002).

Two-layer-plate experiment

After static culture, cells were mixed with pUC18-cm and spread on the bottom layers of selective LB-agar plates for counting the number of transformants or nonselective LB-agar plates for counting viable colonies. Then, the upper
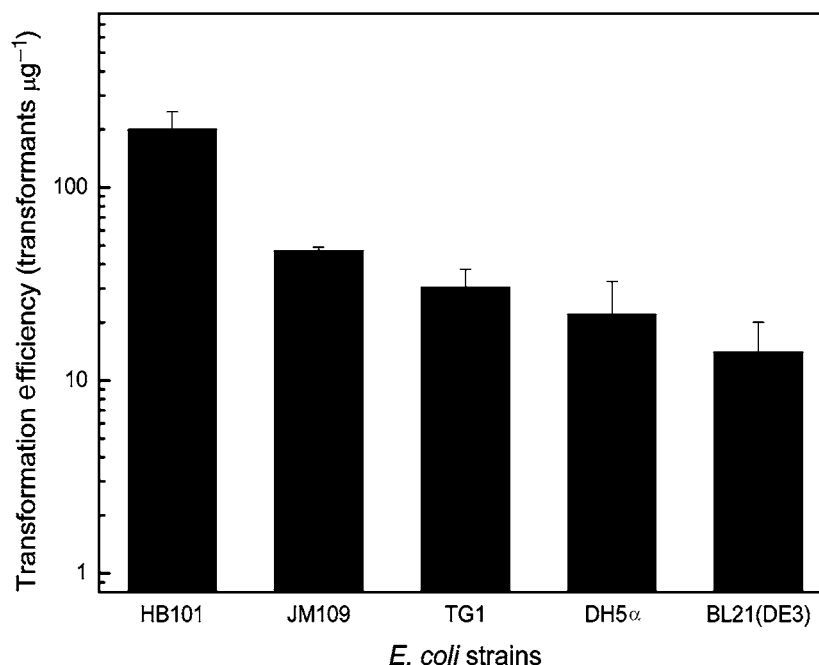
Fig. 1. Transformability of *Escherichia coli* tested with the new protocol. The transformability of five *E. coli* strains [HB101, JM109, TG1, DH5α and BL21 (DE3)] was tested with 2 μg of pDsRED. After 12 h of culture with shaking and 12 h of static culture, plasmid was added to the cultures and the mixtures were spread on plates containing ampicillin. Red transformants were counted. Error bars indicate SDs.

layers of selective and nonselective LB-agar were placed on the bottom layers of selective and nonselective LB-agar plates, respectively. In this experiment, ampicillin and chloramphenicol were solubilized in sterile water and ethanol, respectively. Transformation efficiency and transformation frequency were calculated as previously described.

Results and discussion

A novel transformation system without the standard artificial transformation conditions

Previously, studies of the genetic transformation of *E. coli* have been confined to the classical transformation system in which nonphysiological factors (e.g. a high Ca^{2+} concentration, incubation at a low temperature, a temperature shift in chemical transformation and an electronic shock in electroporation) were necessary (Mandel & Higa, 1970; Hanahan, 1983; Huang & Reusch, 1995). To investigate the natural transformation of *E. coli*, we developed a new transformation system that omitted these factors. The new transformation process was divided into two steps: static culture of stationary-phase cells at 37 °C in LB broth and transformation on LB-agar plates at 37 °C.

The novel transformation system was tested with five strains of *E. coli*. All of the five strains of *E. coli* could be transformed with this transformation system (Fig. 1). The plasmid, which was isolated from red transformants, was digested by restriction enzymes and then electrophoresed. The pattern of the plasmid profile was identical to that of the

donor plasmid pDsRED (data not shown). The transformation efficiency ranged from 14 transformants $μg^{-1}$ in BL21 (DE3) to 197 transformants $μg^{-1}$ in HB101 (Fig. 1). The transformation frequency of HB101 was calculated to be $10^{-6} \sim 10^{-5}$, higher than some naturally transformable bacteria [e.g. *Mycobacterium smegmatis* ($10^{-7} \sim 10^{-6}$) and *Vibrio* sp. strain DI9 (2.0×10^{-7})] (Lorenz & Wackernagel, 1994). Plasmid pSU-A (p15A replicon, amp^r) can also be transferred to *E. coli* in this transformation system, with a transformation efficiency similar to the situation with pDsRED as the donor plasmid (118 ± 13 transformants $μg^{-1}$). We chose to use *E. coli* HB101 in the following experiments. A large amount of DNA is required in our transformation system, and in systems developed by others for assaying transformation of *E. coli* under the low Ca^{2+} concentration condition as well (Tsen *et al.*, 2002; Maeda *et al.*, 2004). So much DNA might be required for the transformation process to take place efficiently. Such a large amount of DNA occurs in biofilms, deep-sea sediments, the human body and soil (Lorenz & Wackernagel, 1994; Molin & Nielsen, 2003; Dell'Anno & Danovaro, 2005).

We compared the transformation efficiency in open systems and closed systems. The transformation efficiency in the open system (74 ± 34 transformants $μg^{-1}$) was about 10-fold higher than that in the closed system (7 ± 3 transformants $μg^{-1}$). After static culture at 37 °C, the volume of culture in an open system reduced from 50 to about $20 \sim 35$ μL by evaporation, while in a closed system the volume did not change. But no significant discrepancy of viable counts was observed in an open system and a closed
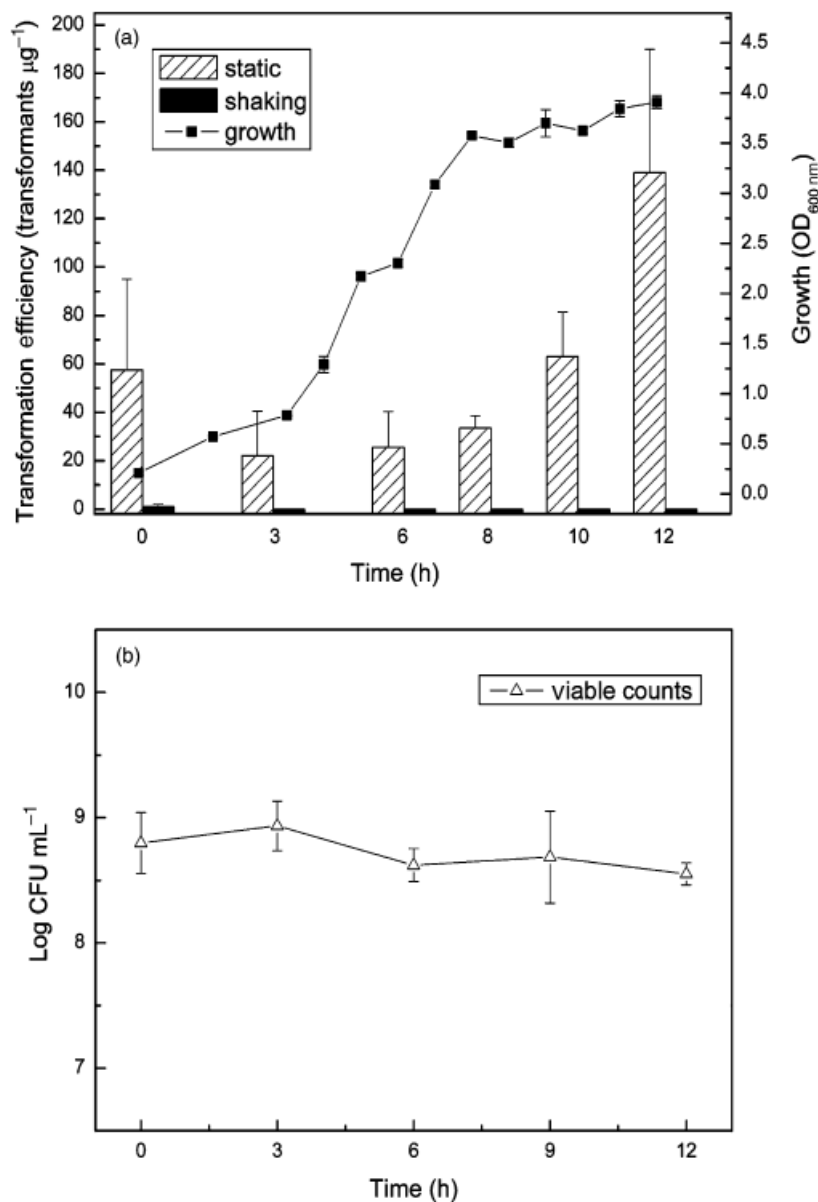
Fig. 2. Effect of 12 h of static culture on transformation. To examine the effect of static culture on transformation, cells at different growth stages (■) were cultured for 12 h with (solid rectangle) or without shaking (shade rectangle) before plating (a). After 12 h of static culturing of cells of different growth stages (a), viable cells were counted (b). The proportion of viable counts per open system was calculated as all of the cells in an open system were spread on one selective plate. Error bars indicate SDs.

system. Compared with that in the closed system, in the open system, many factors (e.g. concentration of oxygen, osmotic changes and the depletion of some nutrients) might be involved in affecting the state of bacteria, which might affect the transformation process.

During the transformation process, no Ca^{2+} was added. Transformation was also not significantly affected by the addition of calcium chelator EGTA (2 mM). The Ca^{2+} concentration in LB-agar plates was much lower than that in the artificial transformation process (Maeda *et al.*, 2004) and is at a level commonly found in the environment. Transformation of *E. coli* under such conditions seems, at the physiological level, to be a natural biological event. As

high Ca^{2+} concentrations and temperature shifts are non-physiological factors and have been extensively studied before (Hanahan, 1983; Baur *et al.*, 1996), we focused on physiological conditions by investigating the effect of static culture in LB broth (without additional Ca^{2+}) at 37 °C on *E. coli* transformation in this new system.

Static culture at 37 °C influences transformation efficiency

In classical transformation, heat shock and incubation at a low temperature or an electronic shock was necessary (Hanahan, 1983). However, in the new transformation
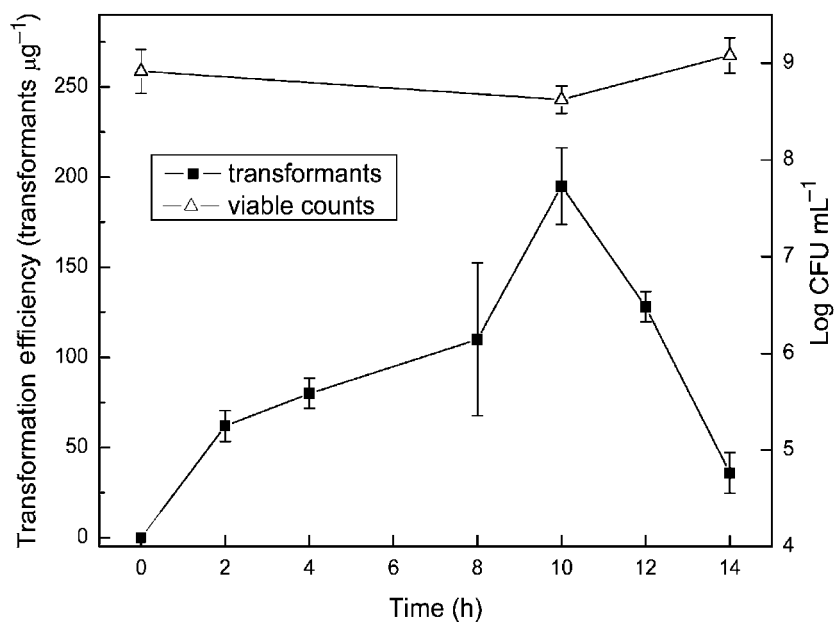
Fig. 3. Optimal static culture time. After 12 h of culture with shaking, bacteria were cultured statically. At intervals, static cultures were supplemented with plasmid before plating. Viable cells (Δ) were counted after static culture to evaluate the effect of static culture on viability. The proportion of viable counts per open system was calculated as all of the cells in an open system were spread on one selective plate. Error bars indicate SDs.

protocol no special conditions were required and the static culture of cells in LB broth at a constant 37 °C was sufficient for transformation. The number of viable bacteria before plating did not change significantly, but few transformants (1 ± 1 transformants) were observed on selective plates if static culture at 37 °C was omitted (Figs 2 and 3). We investigated further how static culture at 37 °C affected the transformation process.

We compared the influence of 12 h of static culture and 12 h of shaking culture on the transformation of bacteria sampled at different stages. The transformation efficiency of bacteria after 3 h of culture with shaking, followed by 12 h of static culture, was 22 ± 13 transformants μg^{-1}. This was the lowest value found among the samples that had been cultured statically for 12 h, but was much higher than the efficiency of bacteria sampled at any stage and cultured subsequently for 12 h with shaking (no more than two transformants μg^{-1}) (Fig. 2a). Transformation efficiencies increased with time in shaken cultures (from 3 h) when the cultures were subsequently statically cultured for 12 h (Fig. 2a). Viability was not significantly changed in cultures to be plated in the experiments (Fig. 2b). These results show that 12 h of static culture significantly increases transformation efficiency. Surprisingly, in the static cultures, the transformation efficiency of the first sample taken at 0 h was as high as that taken at 10 h (Fig. 2a).

As bacteria grown with 12 h of shaking, following by 12 h of static culture, had the highest transformation efficiency (Fig. 2a), we used bacteria cultured for 12 h with shaking to determine the optimal static culture time. Transformation efficiencies varied during static culture. Transformation was undetectable when DNA was added to cultures grown for

12 h with shaking but without subsequent static cultivation and peaked at 10 h after the start of static cultivation (Fig. 3). We repeated this experiment four times and found that the optimal static culture time was between 8 and 12 h. Bacterial viability did not vary significantly during the static culture period (Fig. 3). This shows that the variation in transformation efficiency was not due to bacterial viability, but was influenced by the time in static culture.

Protein synthesis is required for transformation on plates

Static culture was important for transformation, but we did not find any transformants in liquid LB broth during static culture. When static cultures were coincubated with pDsRED and then treated with DNase I, no transformants were observed. After the addition of pDsRED, even if the incubation time in liquid culture before DNase I treatment was extended by 2, 4 or 8 h, respectively, transformants were still not observed if DNase I had been added to the liquid cultures. If the plasmid had entered cells in liquid medium before DNase I treatment, β-lactamase would have been expressed so transformants would have survived on plates containing ampicillin. The absence of transformants after DNase I treatment indicated the failure of DNA uptake in liquid media at least under our experimental conditions (Wang *et al.*, 2002). To confirm whether transformation occurred on plates, we first spread static cultured cells on plates and then spread plasmid DNA directly on these plates. Transformation efficiency with such means was similar to that with our standard protocol. So we concluded that transformation occurred on LB-agar plates. Other investigators have also found that

transformation of *E. coli* is easier on solid media than in liquid media (Tsen *et al.*, 2002; Maeda *et al.*, 2006). Transformation of *E. coli* on solid media but not in liquid media suggested that some factors required for transformation might be induced and function on solid media.

To test whether these factors were *de novo* synthesized proteins, we used plasmid pUC18-cm, which has both ampicillin and chloramphenicol resistance genes. Interestingly, *E. coli* could be transformed with pUC18-cm on LB-agar plates supplemented with ampicillin, but not on LB-agar plates supplemented with chloramphenicol. So we speculate that transformation was inhibited by chloramphenicol. As chloramphenicol inhibits protein synthesis, we deduced that the lack of transformants resulted from the suppression of the synthesis of proteins necessary for transformation (Otto & Silhavy, 2002). Two-layer-plate experiments were used to confirm this speculation. The transformation efficiency of *E. coli* remained between 120 and 165 transformants μg^{-1} if the upper layers contained ampicillin (Fig. 4). These results demonstrate that ampicillin in the upper layer affected transformation efficiency very little. However, when the upper layer containing chloramphenicol was added at intervals, transformation efficiencies increased over time and reached a plateau (Fig. 4). In this experiment, no significant fluctuation in bacterial viability was observed (Fig. 4). These results demonstrate that the transformation process could be interrupted by the presence of chloramphenicol in the upper layer. The inability of plasmid pUC-cm to transfer a Cm^r gene is not due to an inherent inability of *E. coli* to maintain the plasmid, as it can be artificially transformed into *E. coli* or naturally transformed into *E. coli* containing an Amp^r marker and subsequently maintained. We suggest that chloramphenicol

interrupted transformation by preventing the plasmid entering cells through inhibition of the synthesis of proteins required for DNA uptake (Reeve *et al.*, 1984). When the chloramphenicol-containing upper layer was plated after 9 h of incubation, there were no effects on transformation efficiency, thus demonstrating that the transformation process was completed within 9 h.

De novo synthesized protein required for transformation on plates implied that specific genes might be involved in the transformation process. This further confirms that *E. coli* is naturally transformable. Putative *E. coli* competence genes have been suggested by genomic analysis (Claverys & Martin, 2003). With our natural transformation protocol, it would be possible to determine whether these genes actually affect natural transformation in *E. coli* by gene inactivation and overexpression combined with mutational analysis (Aas *et al.*, 2002). Current data show that the absence of *yhiR* gene, required for DNA as a nutrient (Finkel & Kolter, 2001; Palchevskiy & Finkel, 2006), enhances transformation efficiency (data not shown).

In this study, we have demonstrated the natural transformability of *E. coli* in a new procedure in which cells are cultured statically at a constant 37 °C and where protein synthesis is necessary for transformation on plates. We propose that natural competence induction of *E. coli* in this new system requires two integrated steps: early competence induction during static culture and late competence induction on plates and that transformation process occurs on plates. Showing the natural transformability of *E. coli* could be the first step in discovering the mechanism of natural transformation in this species. Using this simple protocol, we will attempt to identify competence genes in *E. coli*. We also anticipate that the protocol developed here could be
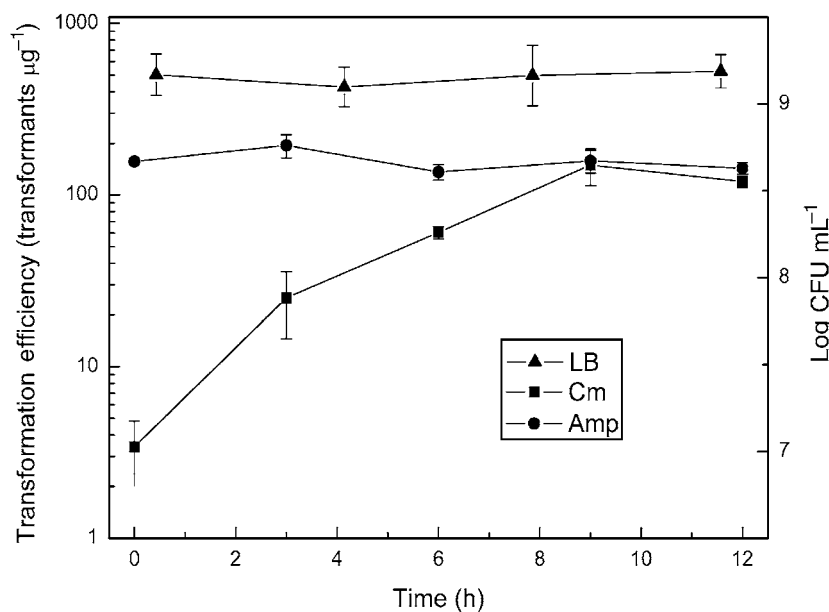
Fig. 4. Effect of chloramphenicol on transformation of *Escherichia coli* HB101 in the novel system. Mixtures of static culture and plasmid were spread on the bottom layer of LB-agar with ampicillin. At intervals, second layers of LB-agar with ampicillin (●) or chloramphenicol (■) were placed on the bottom layer. Transformants at the layer interface were observed and counted. Viable counts (▲) at the layer interface of LB plates without antibiotics. Error bars indicate SDs. LB, LB-agar plates; Amp, LB-agar plates supplemented with ampicillin; Cm, LB-agar plates supplemented with chloramphenicol.

used to demonstrate the natural transformability of other bacterial species. Considering both the amount of DNA available from various sources in the environment and the natural transformability of *E. coli*, using this bacterium as a genetic engineering recipient could have serious environmental consequences (Lorenz & Wackernagel, 1994; Droge *et al.*, 1998; Dell'Anno & Danovaro, 2005).

Acknowledgements

References

Aas FE, Wolfgang M, Frye S, Dunham S, Lovold C & Koomey M (2002) Competence for natural transformation in *Neisseria gonorrhoeae*: components of DNA binding and uptake linked to type IV pilus expression. *Mol Microbiol* **46**: 749–760.

Baur B, Hanselmann K, Schlimme W & Jenni B (1996) Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. *Appl Environ Microbiol* **62**: 3673–3678.

Claverys JP & Martin B (2003) Bacterial 'competence' genes: signatures of active transformation, or only remnants? *Trends Microbiol* **11**: 161–165.

Dell'Anno A & Danovaro R (2005) Extracellular DNA plays a key role in deep-sea ecosystem functioning. *Science* **309**: 2179.

Dennies JJ & Zylstra GJ (1998) Plasposons: modular self-cloning minitransposon derivatives for rapid genetic analysis of gram-negative bacterial genomes. *Appl Environ Microbiol* **64**: 2710–2715.

Droge M, Puhler A & Selbitschka W (1998) Horizontal gene transfer as a biosafety issue: a natural phenomenon of public concern. *J Biotechnol* **64**: 75–90.

Finkel SE & Kolter R (2001) DNA as a nutrient: novel role for bacterial competence gene homologs. *J Bacteriol* **183**: 6288–6293.

Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**: 557–580.

Huang R & Reusch RN (1995) Genetic competence in *Escherichia coli* requires poly-beta-hydroxybutyrate/calcium polyphosphate membrane complexes and certain divalent cations. *J Bacteriol* **177**: 486–490.

Li WH, Guo PY, Xie ZX & Shen P (2001) *Escherichia coli* absorbing external-DNA under condition of lower Ca^{2+} concentration. *J WuhanUniv (Nat Sci Ed)* **47**: 247–250.

Lorenz MG & Wackernagel W (1994) Bacterial gene transfer by natural genetic transformation in the environment. *Microbiol Rev* **58**: 563–602.

Maeda S, Sawamura A & Matsuda A (2004) Transformation of colonial *Escherichia coli* on solid media. *FEMS Microbiol Lett* **236**: 61–64.

Maeda S, Ito M, Ando T, Ishimoto Y, Fujisawa Y, Takahashi H, Matsuda A, Sawamura A & Kato S (2006) Horizontal transfer of nonconjugative plasmids in a colony biofilm of *Escherichia coli*. *FEMS Microbiol Lett* **255**: 115–120.

Mandel M & Higa A (1970) Calcium-dependent bacteriophage DNA infection. *J Mol Biol* **53**: 159–162.

Molin S & Tolker-Nielsen T (2003) Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilization of the biofilm structure. *Curr Opin Biotechnol* **14**: 255–261.

Otto K & Silhavy TJ (2002) Surface sensing and adhesion of *Escherichia coli* controlled by the Cpx-signaling pathway. *Proc Natl Acad Sci USA* **99**: 2287–2292.

Palchevskiy V & Finkel SE (2006) *Escherichia coli* competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient. *J Bacteriol* **188**: 3902–3910.

Reeve CA, Amy PS & Matin A (1984) Role of protein synthesis in the survival of carbon-starved *Escherichia coli* K-12. *J Bacteriol* **160**: 1041–1046.

Solomon JM & Grossman AD (1996) Who's competent and when: regulation of natural genetic competence in bacteria. *Trends Genet* **12**: 150–155.

Tolker-Nielsen T, Brinch UC, Ragas PC, Andersen JB, Jacobsen CS & Molin S (2000) Development and dynamics of *Pseudomonas* sp. biofilms. *J Bacteriol* **182**: 6482–6489.

Tsen SD, Fang SS, Chen MJ, Chien JY, Lee CC & Tsen DHL (2002) Natural plasmid transformation in *Escherichia coli*. *J Biomed Sci* **9**: 246–252.

Wang Y, Goodman SD, Redfield RJ & Chen C (2002) Natural transformation and DNA uptake signal sequences in *Actinobacillus actinomycetemcomitans*. *J Bacteriol* **184**: 3442–3449.

Xie ZX, Liu Y, Chen XD, Shen P & Qu SS (2000) Thermochemical studies on the competence development of *Escherichia coli* HB101. *Acta Chimica Sinica* **58**: 153–156.

Supplementary material

The following supplementary material is available for this article:

Fig. S1. Effect of oxygen or nitrogen on transformation.

Fig. S2. Two-layer-plate assay with the standard Ca^{2+} transformation protocol.

Fig. S3. Effect of ethanol and EGTA on transformation.

Table S1. Main difference between our system and that in Tsen *et al.* (2002).

http://www.blackwell-synergy.com/doi/abs/10.1111/j.1574-6968.2006.00503.x (This link will take you to the article abstract).

Supplemental Materials

Figure 1
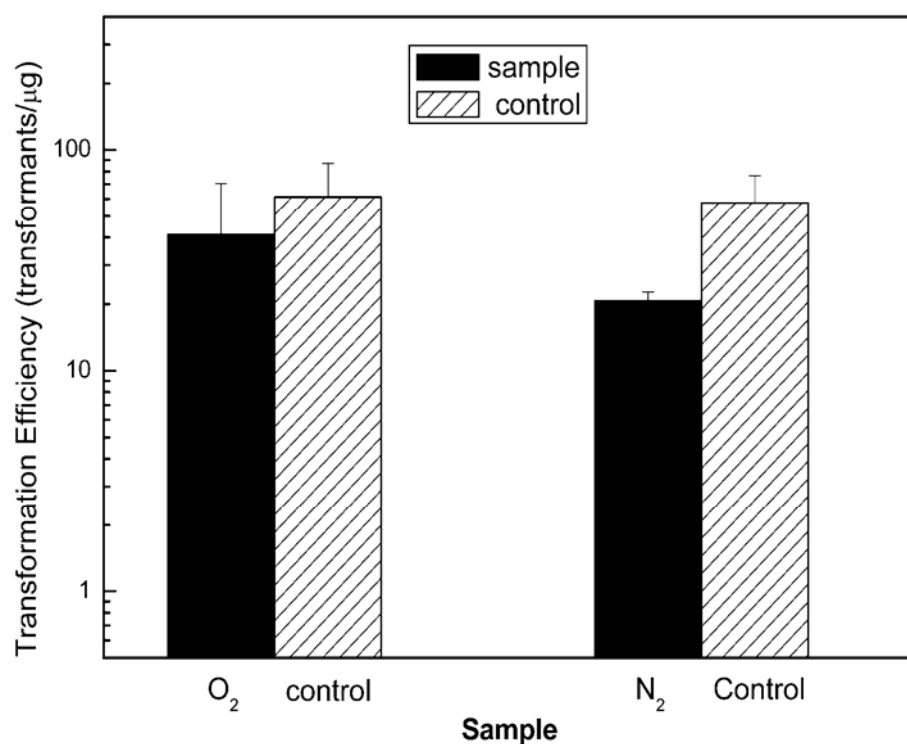
Figure 1 Effect of oxygen or nitrogen on transformation.

Open systems were placed in an anaerobic jar full of oxygen (solid rectangle, left column) or nitrogen (solid rectangle, right column). All other procedures were identical to the control treated according to the standard natural transformation protocol mentioned in our manuscript. Viable counts were counted and no significant discrepancy was detected.

Figure 2
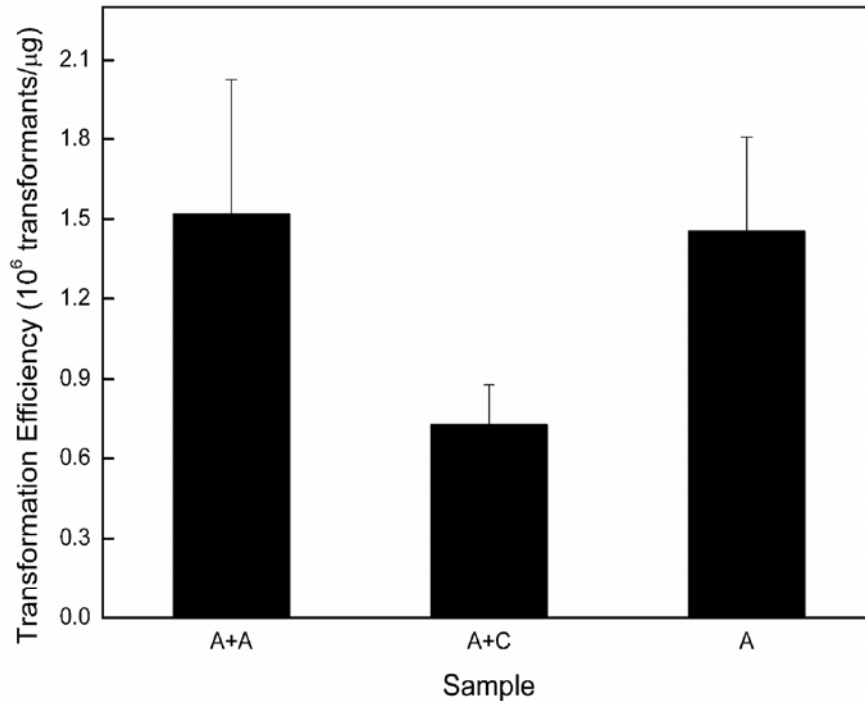
Figure 2 Two-layer-plate assay with the standard Ca^{2+} transformation protocol.

Initially, transformed cells were spread on LB-agar plates containing ampicillin.

Then LB-agar plates containing ampicillin or chloramphenicol were covered on the

bottom layer. LB-agar plates containing ampicillin without upper layer as the control

to evaluate the effect of addition of upper layer. A+A: bottom layer with ampicillin

and upper layer with ampicillin; A+C: bottom layer with ampicillin and upper layer

with chloramphenicol. A: bottom layer with ampicillin but without upper layer.

Figure 3
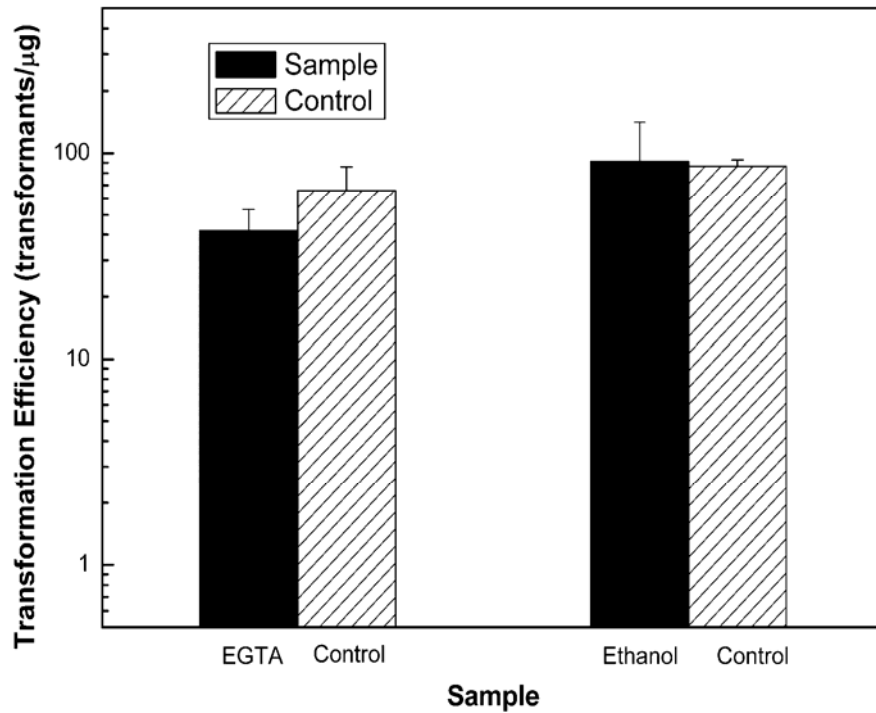
Figure 3 Effect of ethanol and EGTA on transformation.

Except for the addition of EGTA (2 mM) (shade rectangle, left columns) or of 100 μl of ethanol (solution of chloramphenicol) (shade rectangle, right columns) to LB-agar containing ampicillin, all other procedures were identical to the control (solid rectangle, left and right columns) treated according to the standard natural transformation protocol mentioned in our manuscript.

Main differences between our system and that in Tsen *et al*. 2002:

	Tsen *et al*. 2002	Ours
Low temperature incubation	Stationary cells stored at 4°C	Stationary cells static cultured at 37°C
Heat shock	Not mentioned	No
Culture media	M9 medium	LB broth or LB-agar plates
Static incubation	Not mentioned	Required
De novo protein synthesis	Not mentioned	Might be Required
Transformation efficiency (with 2 µg of plasmid)	$10^{-7} \sim 10^{-8}$	$10^{-5} \sim 10^{-6}$
The method for transferring cells from liquid culture to plates	Spot	Spread

Technical Annex

Figure 1. Transformation protocol

Figure 1. Transformation protocol
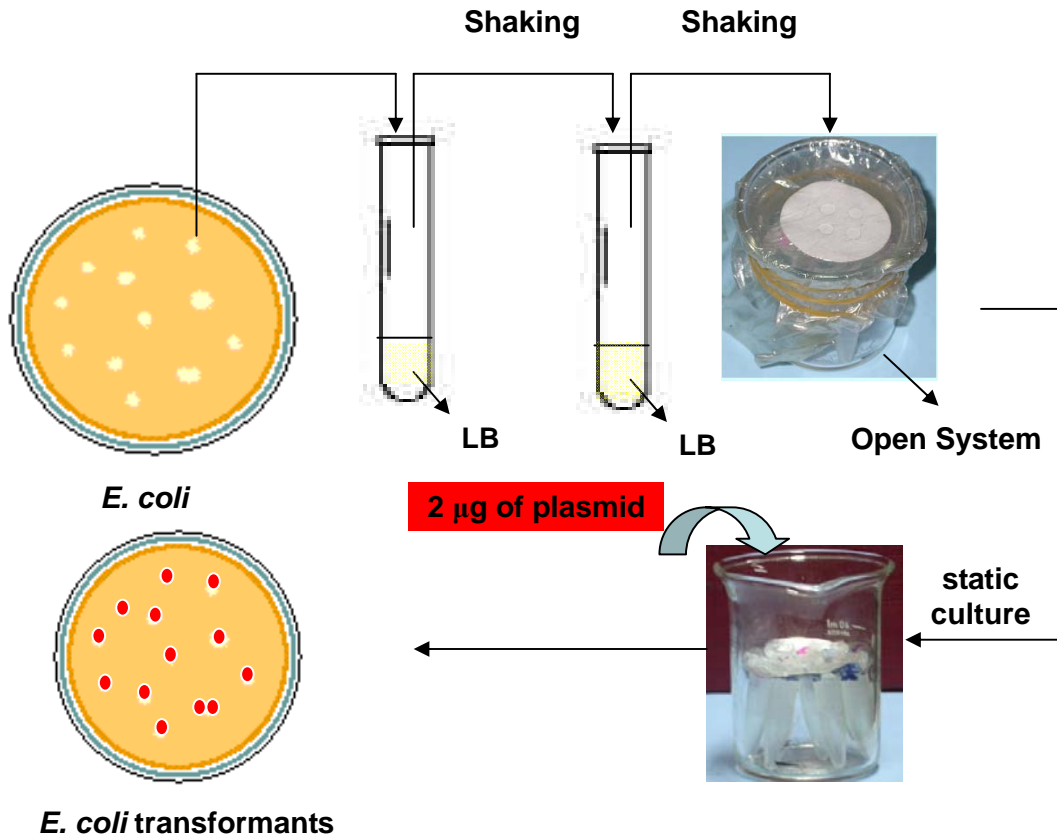
All procedures were performed at 37°C. A single colony was picked up from a LB-agar plate and inoculated into a glass tube containing 5 ml of fresh LB broth. After overnight incubation, 50 μl of the culture were transferred to another glass tube containing 5 ml of fresh LB broth. After 14 h of horizontal shaking incubation, 50 μl of the culture were transferred to a plastic tube, which was placed in a beaker covered by an air permeable membrane (or 1 ml of the culture was transferred to a glass beaker covered by an air permeable membrane), followed by further incubation in the beaker statically for 14 h. After static culture in the beaker, 2 μg of the plasmid pDsRED was added to 40-50 μl of the static culture and mixed gently but thoroughly. The mixture was plated onto a selective LB-agar plate, which had been air dried at 37°C for one day. The number of transformants on plates was counted after overnight incubation.

Table 1. Suggestions for improvement of spontaneous plasmid transformation of *E. coli* cells on solid medium.

Inoculation	Use preferably freshly streaked colonies (do not to use colonies stored in the refrigerator for more than 2 weeks)
Culture with shaking	Cells should be cultured with horizontal shaking rather than rotating shaking
Static culture in beaker or in tubes	The optimal time for static culture varies depending on the environment (*e. g.* humidity, air pressure). Normally after static culture in the open system, the volume of leftover should be about half of the original input.
OD_{600} after static culture	After static culture in beakers, OD_{600} is 4.5 ~ 4.9. Plating 40-50 μl of the culture with 2 μg of pDsRED normally yields 50-100 transformants per plate.
Plates preparing	For each plate, use 20 ml of LB-agar (9% Kalys agar or 5% Bacto-Difco agar) for plates with a diameter of 8.5 cm and 10 ml for plates with a diameter of 6 cm. Agar powders from different companys are not equivalent, i.e. the origin of agar can strongly affect transformation efficiency.
Plates	It is recommended to place LB-agar plates at 37°C for 24 hrs and then use them immediately (recommended) or keep them at room temperature for less than 3 days.
Plasmid store	It is recommended to prepare fresh plasmid immediately before (in 2 weeks) the transformation experiment and temporarily store plasmid in 4°C. Plasmid can be stored in -70°C without frequent freezing-melting within less than 2 month. It is not recommended to store plasmid in -20°C.
Plasmid form	Only circular plasmid contributes to transformation. Linear plasmid does not contribute to transformation. So, it is necessary to check plasmid integrity by gel electrophoresis of plasmid preparation.
Spreading	To perform plating, it is preferable to use glass beads rather than rakes.
Culture	Transformants can also be obtained by using M9 culture plus glucose.
Strains	*E. coli* strains RR1 (K12 × B), ZK126 (W3110 derivative), MC4100 (MG1655 derivative) often display a transformation frequency of ~10^{-6}. Transformation frequency in other *E. coli* strains could be lower (*e.g.* transformation frequency in BL21 is ~10^{-7}).

Chapter 2

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

This work was published (**Sun, *et al*.** 2009. Transforming DNA uptake gene orthologs do not mediate spontaneous plasmid transformation in *Escherichia coli*. J Bacteriol **191:**713-9) and is reproduced hereafter.

The reprint is followed by Supplemental Material in which I document and discuss the possible involvement of the putative key regulator of *E. coli* competence Sxy (see Introduction) in plasmid transformation on solid medium (Table S1). In addition, I report and discuss results of my attempts at transforming *E. coli* cells on filters placed on the surface of LB plates (Table S2).

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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† Supplemental material for this article may be found at http://jb.asm.org/.
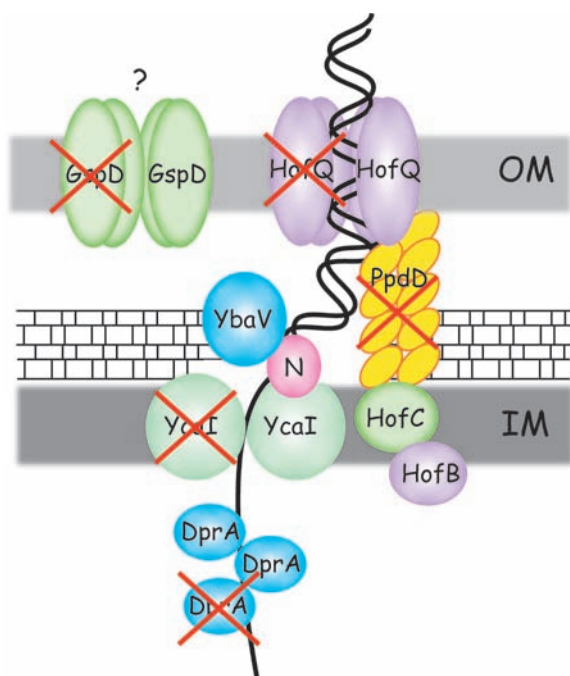▽ Published ahead of print on 14 November 2008.

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 $1 \times$ ~10^{-5} and ~4×10^{-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	CGTACCCGCTTGATAAATGTTTCCGTCGGGGAACTTACAGGAATGAATGgtgtaggctggagctgcttc; *gspD*; (−59, −10)	This study
GSPD H2P2	TACGGTGAGTGAATTCTCATATGAATGCCTCACCGTGACGATGGCGCAGGcatatgaatatcctccttag; *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	ATCACTGACCAATCGCAAAGATTGCTAAGGCTGCTTATGGCAGGGAGATAcatatgaatatcctccttag; *dprA* (*smf*); (−53, −4)	22
SMF/DPRA H2P2	GCTGCGATCCATCCTGCTAACTCCAGTTCGAGTAGTTGAGTAACTACCTCgtgtaggctggagctgcttc; *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 A̲TG 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 addi-

tion 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+}.

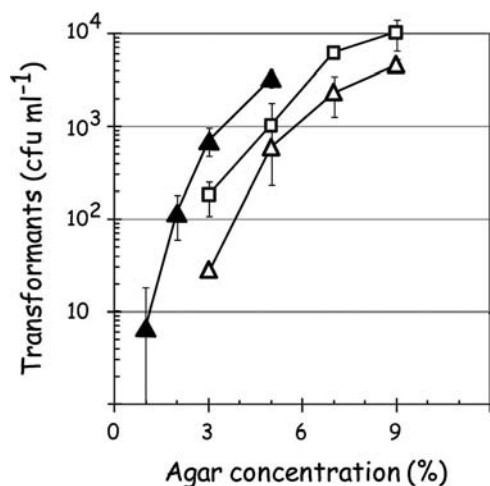Nevertheless, the experiment in Fig. 3A did not rule out the

FIG. 2. Effect of agar concentration on plasmid transformation efficiency. A total of 40 μl (~8 × 10⁷ 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).
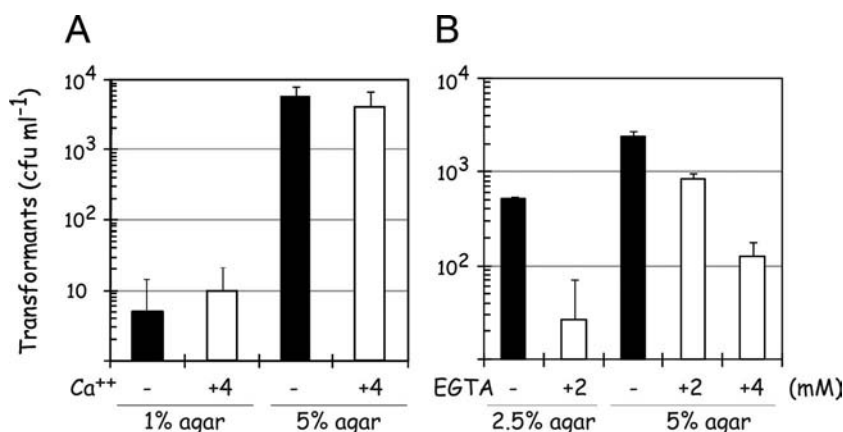
The addition of EGTA resulted in a decrease in transformation frequencies on agarose plates stronger than that observed with agar plates (compare Fig. S2B in the supplemental material with Fig. 3B). These data would be consistent with the requirement for a cation whose concentration is lower in agarose 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 × 10⁶ and 1.28 × 10⁸ 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 × 10⁷ 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.
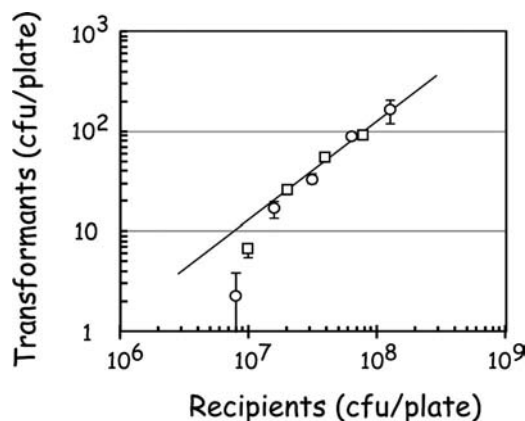
FIG. 4. Effect of cell density on plasmid transformation efficiency. Twofold serial dilutions from a ZK126 culture that had been incubated under static conditions were mixed with pDsRED plasmid DNA (see Materials and Methods), and 50 μl was immediately spread onto selective plates containing 5% agar. 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
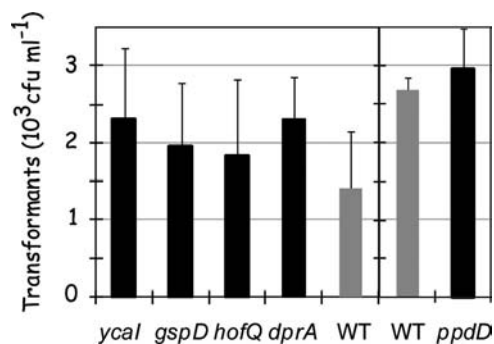
genes for uptake of ssDNA exist in the *E. coli* genome, we propose that plasmid DNA enters *E. coli* cells in the form of dsDNA molecules. Such a mechanism would be fully consistent with the observed dispensability of DprA (Smf), a protein normally required for protection of internalized ssDNA.

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 Ca²⁺ 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 rang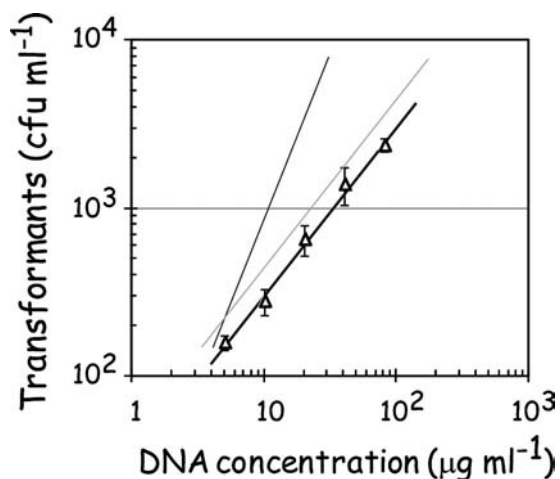ing from 6.25 to 100 $\mu g\ ml^{-1}$) were added to 40-μl aliquots from a ZK126 culture, prepared as described in Materials and Methods. Each aliquot was then spread on selective plates containing 9% agar. Gray and black lines indicate slopes of 1 and 2, respectively.

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 trans-

formation 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.

ACKNOWLEDGMENTS

REFERENCES

1. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2:**2006.
2. **Bartfai, T.** 1979. Preparation of metal-chelate complexes and the design of steady-state kinetic experiments involving metal nucleotide complexes. Adv. Cyclic Nucleotide Res. **10:**219–242.
3. **Baur, B., K. Hanselmann, W. Schlimme, and B. Jenni.** 1996. Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. Appl. Environ. Microbiol. **62:**3673–3678.
4. **Bergé, M., I. Mortier-Barrière, B. Martin, and J. P. Claverys.** 2003. Transformation of *Streptococcus pneumoniae* relies on DprA- and RecA-dependent protection of incoming single strands. Mol. Microbiol. **50:**527–536.
5. **Blokesch, M., and G. K. Schoolnik.** 2008. The extracellular nuclease Dns and its role in natural transformation of *Vibrio cholerae*. J. Bacteriol. **190:**7232–7240.
6. **Blomqvist, T., H. Steinmoen, and L. S. Håvarstein.** 2006. Natural genetic transformation: a novel tool for efficient genetic engineering of the dairy bacterium *Streptococcus thermophilus*. Appl. Environ. Microbiol. **72:**6751–6756.
7. **Cameron, A. D., and R. J. Redfield.** 2006. Non-canonical CRP sites control competence regulons in *Escherichia coli* and many other gamma-proteobacteria. Nucleic Acids Res. **34:**6001–6014.
8. **Chen, I., and D. Dubnau.** 2004. DNA uptake during bacterial transformation. Nat. Rev. Microbiol. **2:**241–249.
9. **Claverys, J. P., and B. Martin.** 2003. Bacterial "competence" genes: signatures of active transformation, or only remnants? Trends Microbiol. **11:**161–165.
10. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA **97:**6640–6645.
11. **Finkel, S. E., and R. Kolter.** 2001. DNA as a nutrient: novel role for bacterial competence gene homologs. J. Bacteriol. **183:**6288–6293.
12. **Francetic, O., D. Belin, C. Badaut, and A. P. Pugsley.** 2000. Expression of the endogenous type II secretion pathway in *Escherichia coli* leads to chitinase secretion. EMBO J. **19:**6697–6703.
13. **Karudapuram, S., X. Zhao, and G. J. Barcak.** 1995. DNA sequence and characterization of *Haemophilus influenzae dprA*$^+$, a gene required for chromosomal but not plasmid transformation. J. Bacteriol. **177:**3235–3240.
14. **Maeda, S., A. Sawamura, and A. Matsuda.** 2004. Transformation of colonial *Escherichia coli* on solid media. FEMS Microbiol. Lett. **236:**61–64.
15. **Martell, A. E., and R. M. Smith.** 1974. Critical stability constants. Plenum Press, New York, NY.
15a. **Mirończuk, A. M., A. T. Kovács, and O. P. Kuipers.** 2008. Induction of natural competence in *Bacillus cereus* ATCC14579. Microb. Biotech. **1:**226–235.
16. **Mortier-Barrière, I., M. Velten, P. Dupaigne, N. Mirouze, O. Piétrement, S. McGovern, G. Fichant, B. Martin, P. Noirot, E. Le Cam, P. Polard, and J. P. Claverys.** 2007. A key presynaptic role in transformation for a widespread bacterial protein: DprA conveys incoming ssDNA to RecA. Cell **130:**824–836.
17. **Notani, N. K., J. K. Setlow, D. McCarthy, and N.-L. Clayton.** 1981. Transformation of *Haemophilus influenzae* by plasmid RSF08885. J. Bacteriol. **148:**812–816.
18. **Palchevskiy, V., and S. E. Finkel.** 2006. *Escherichia coli* competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient. J. Bacteriol. **188:**3902–3910.
19. **Redfield, R. J., A. D. Cameron, Q. Qian, J. Hinds, T. R. Ali, J. S. Kroll, and P. R. Langford.** 2005. A novel CRP-dependent regulon controls expression of competence genes in *Haemophilus influenzae*. J. Mol. Biol. **347:**735–747.
20. **Renelli, M., V. Matias, R. Y. Lo, and T. J. Beveridge.** 2004. DNA-containing membrane vesicles of *Pseudomonas aeruginosa* PAO1 and their genetic transformation potential. Microbiology **150:**2161–2169.
21. **Saunders, C. W., and W. R. Guild.** 1981. Monomer plasmid DNA transforms *Streptococcus pneumoniae*. Mol. Gen. Genet. **181:**57–62.
22. **Smeets, L. C., S. C. Becker, G. J. Barcak, C. M. Vandenbroucke-Grauls, W. Bitter, and N. Goosen.** 2006. Functional characterization of the competence protein DprA/Smf in *Escherichia coli*. FEMS Microbiol. Lett. **263:**223–228.
23. **Sun, D., Y. Zhang, Y. Mei, H. Jiang, Z. Xie, H. Liu, X. Chen, and P. Shen.** 2006. *Escherichia coli* is naturally transformable in a novel transformation system. FEMS Microbiol. Lett. **265:**249–255.
24. **Tsen, S. D., S. S. Fang, M. J. Chen, J. Y. Chien, C. C. Lee, and D. H. Tsen.** 2002. Natural plasmid transformation in *Escherichia coli*. J. Biomed. Sci. **9:**246–252.

Supplemental Material 1

Supplemental Discussion

The DNA uptake machinery of the Gram-negative prototype *H. influenzae*

More than 20 genes belonging to the CRP-S regulon (defined as genes preceded by canonical Sxy-dependent CRP sites) have been reported to be required for DNA binding and uptake in *H. influenzae* (11). However, an experimental demonstration of their involvement in transformation is not always available.

A comprehensive view of the situation and relevant references can be found in Fig. 1 in reference (17). To summarize, a role in transformation in *H. influenzae* has been demonstrated only for ComE and ComF (15, 16), DprA (9), and Rec2 (5) (Table S1). In addition, the finding that cassette insertions in the *pilABCD* operon affected transformation suggested the presence of at least one transformation-related gene in the operon but polar effects prevented individual characterization (7). Insertions in the *comABCDEF* operon were similarly reported to affect transformation (15) but as the last two genes are required for transformation, no conclusion could be drawn regarding the possible involvement of the first four genes in the operon.

The putative DNA uptake machinery of *E. coli*

Two sets of *E. coli* genes potentially involved in DNA uptake were identified as homologs of genes suspected or demonstrated to be required for natural transformation in the Gram-negative *H. influenzae* (8, 4) (Table S1). Both sets of genes have been proposed to belong to the competence regulon of *E. coli* (2).

The first set of *E. coli* genes included *hofQ*, *yrfABCD*, *yhiR*, and *yhgHI* (8). Although the latter was described (5) as the homolog of genes shown to play a role competence and transformation in *H. influenzae* (*comG*, also called *orfG*) and in *Neisseria gonorrhoeae* (NGO1426), no data exist to establish their involvement in either species (17). The possible involvement of these genes in the uptake of exogenous DNA in *E. coli* has only been documented through the characterization of the use of DNA as a nutrient (8, 10). After 24 h of incubation in minimal medium supplemented with 1 mg ml^{-1} ultrapure sonicated salmon sperm DNA as the sole source of carbon and energy, a growth yield (defined as the number of cells after incubation divided by the number of cells at inoculation) of 120 was reported for

wildtype *E. coli* cells, whereas the various mutants displayed a growth yield varying from 0.84-0.85 (*hghIR*), to 2.1-2.5 (*yrfABCD*), and to 3.2-3.6 (*yhiR* and *hofQ*) [Fig. 3 in reference (10)]. These mutants also displayed a loss of competitive fitness during long-term stationary-phase incubation with wildtype cells (10), a phenotype attributed to their inability to compete for extracellular DNA as a nutrient resource (8). Nevertherless, with the exception of *hofQ*, the function of these genes remains elusive.

A second set of *E. coli* genes comprises the homologs of the *B. subtilis* DNA uptake machinery, *ppdD-hofBC*, *pppA*, *ybaV*, and *ycaI* (4). These genes potentially encode a pseudopilin providing access of DNA to its receptor through the peptidoglycan (PpdD) (12), a prepilin peptidase (PppA), a traffic NTPase (HofB) and a polytopic membrane protein (HofC) required for formation of the transformation pseudopilus (3), a periplasmic DNA receptor (YbaV), and a transmembrane channel to cross the inner membrane (YcaI) (Table S1). So far, it has not been established whether these genes are involved in DNA uptake or even if they are required for the use of DNA as a nutrient by *E. coli* cells.

TABLE S1. *E. coli* **orthologues of proteins involved in DNA uptake (and processing) in** *H. influenzae* **and** *B. subtilis* (see Supplemental Discussion)

Names in red correspond to proteins with a demonstrated role in DNA uptake or transformation (*B. subtilis* and *H. influenzae*) or in "nutritional" DNA uptake (*E. coli*)
Names in green correspond to proteins encoded in operons in which at least one gene is required for transformation
Names in black correspond to proteins for which no data exist to indicate involvement in transformation

Protein	*E. coli*	ref.	other name	ref.	*H. influenzae*	ref.	other name	ref.	other name	ref.	other name	ref.	*B. subtilis*	ref.
The outer membrane channel														
Secretin	HofQ[a]	(8)			ComE[b]	(4)	HI0435				(PilQ)[@]		n.a.	
The competence pseudopilus														
Pilins or pseudopilins	PpdD[c]	(12, 4)			PilA[d]	(17, 11)	HI0299	(4)					ComGC[e]	(3)
													ComGD[e]	(3)
													ComGE[e]	(3)
													ComGG[e]	(3)
?	HofM[a]	(12)	YrfD	(10)			HI0439		ComA[b]	(8, 10, 17, 11)	(PilM)[@]			
	HofN[a]	(12)	YrfC	(10)			HI0438		ComB[b]	(8, 10, 17, 11)	(PilN)[@]			
	HofO[a]	(12)	YrfB	(10)			HI0437		ComC[b]	(8, 10, 17, 11)	(PilO)[@]			
	HofP[a]	(12)	YrfA	(10)			HI0436		ComD[b]	(8, 10, 17, 11)	(PilP)[@]			
?	GntX		YhgH	(8, 10)			HI0434		ComF[b]	(8, 10, 17, 11)	Com101A		ComFC[f #]	(3)
	GntY		YghI*	(8, 10)			HI0433*		ComG*	(8, 10, 17, 11)			n.a.	
Prepilin peptidase	PppA	(4)			PilD[d]	(17, 11)	HI0296	(4)					ComC	(3)
Traffic NTPase	HofB[c]	(4)			PilB[d]	(17, 11)	HI0298	(4)					ComGA[e]	(3)
Polytopic membrane protein	HofC[c]	(4)			PilC[d]	(17, 11)	HI0297	(4)					ComGB[e]	(3)
DNA translocation machinery														
DNA receptor	YbaV	(4)			ComE1	(11)	HI1008	(4)					ComEA[g]	(3)
(Inner) membrane channel	YcaI	(4)			Rec2	(4)	HI0061						ComEC[g]	(3)
DNA translocase	n.i.				n.i.								ComFA[f]	(3)
DNA processing														
ssDNA binding and RecA loading	Smf				DprA	(9)	HI0985						Smf	

n.a., not applicable; n.i., not identified

[a] *hofMNOPQ* operon

[b] *comABCDEF* operon

[c] *ppdD-HofBC* operon

[d] *pilABCD* operon

[e] *comG* [*ABCDEG*] operon

[f] *comF* [*ABC*] operon

[@] Name of *Neisseria gonorrhoeae* homologs

[#] Inactivation of *comFC* was reported to reduce transformation by 5 to 10-fold, to have no effect on DNA binding and only a limited effect on DNA uptake. The exact role of ComFC thus remains unknown

[g] *comE* [*AC*] operon

* Although *yhgHI* (*gntY*) was described (10) as a homolog of a gene shown to play a role in *H. influenzae* (*comG*), no data exist to indicate involvement in competence and transformation of this gene (also called *orfG*) in this species (17)
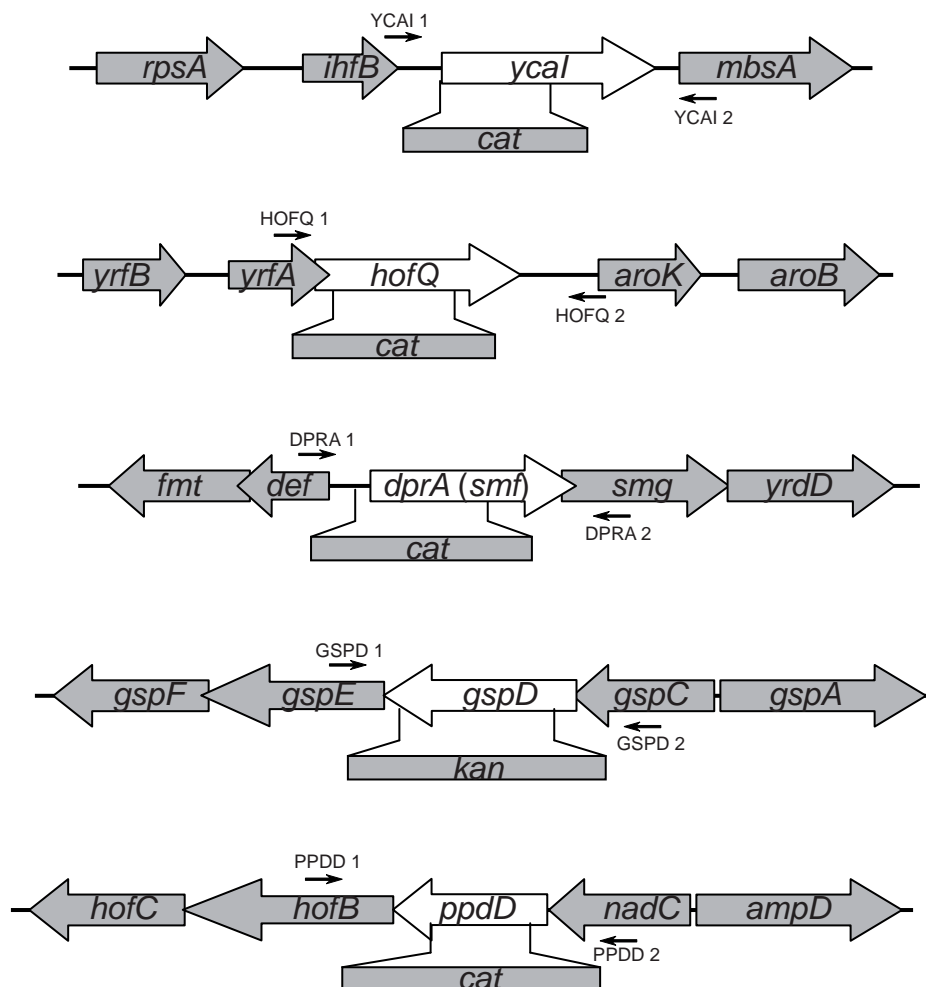
FIG. S1. Control of the structure of *E. coli* mutant strains generated in this work (see accompanying Table S2). (A) Genetic organization of the various chromosomal regions and location of cassette insertion mutants generated in this study.

B
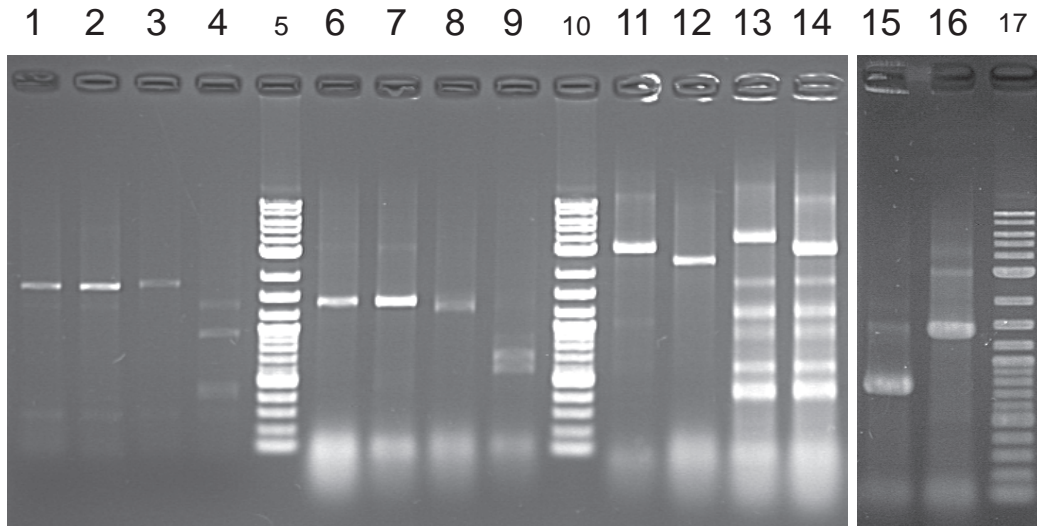
FIG. S1. Control of the structure of _E. coli_ mutant strains generated in this work (see accompanying Table S2). (B) Analysis of PCR fragments for confirmation of the structure of _E. coli_ mutant strains. From left to right, each pair of lanes compares wildtype and mutant structures, as follows.

Lanes:

1, wild type; 2, _hofQ_ mutant; 3, _Xba_I-digested wild type; 4, _Xba_I-digested _hofQ_ mutant

6, wild type; 7, _dprA_ mutant; 8, _Pvu_II-digested wild type; 4, _Pvu_II-digested _dprA_ mutant

11, wild type; 12, _gspD_ mutant

13, wild type; 14, _ycaI_ mutant

15, wild type; 16, _ppdD_ mutant

5, 10, and 17, 2-Log DNA ladder

TABLE S2. Prediction of the structure of *E. coli* mutant strains generated in this work
(see accompanying Fig. S1A)

Lane[a]	Strain[b]	Primer pair[b]	Restriction enzyme	Size[c] (kb)
1	ZK126	HOFQ 1-HOFQ 2	-	1.80
2	ZK126 *hofQ*::cat	HOFQ 1- HOFQ 2	-	1.77
3	ZK126	HOFQ 1- HOFQ 2	*Xba*I	1.80
4	ZK126 *hofQ*::cat	HOFQ 1-HOFQ 2	*Xba*I	0.39 0.45 0.93
5	2-Log marker			
6	ZK126	SMF/DPRA 1-SMF/DPRA 2	-	1.36
7	ZK126 *dprA*::cat	SMF/DPRA 1-SMF/DPRA 2	-	1.23
8	ZK126	SMF/DPRA 1-SMF/DPRA 2	*Pvu*II	0.11 1.25
9	ZK126 *dprA*::cat	SMF/DPRA 1-SMF/DPRA 2	*Pvu*II	0.09 0.53 0.61
10	2-Log marker			
11	ZK126	GSPD 1-GSPD 2	-	3.02
12	ZK126 *gspD*::kan	GSPD 1-GSPD 2	-	2.51
13	ZK126	YCAI 1-YCAI 2	-	3.46
14	ZK126 *ycaI*::cat	YCAI 1-YCAI 2	-	2.70
15	ZK126	PPDD 1-PPDD 2	-	0.68
16	ZK126 *ppdD*::cat	PPDD 1-PPDD 2	-	1.30
17	2-Log marker			

[a] Lane numbers refer to Fig. S1B

[b] Strains and PCR primers are listed in Table S2

[c] Sizes predicted on the basis of available DNA sequence information for PCR fragments generated with primer pairs indicated in the third column. When the predicted sizes of wildtype and mutant PCR fragments were too close, the fragments were discriminated through digestion with the indicated restriction enzyme

Sizes of bands observed on the gels shown in Fig. S1B are in good agreement with the prediction
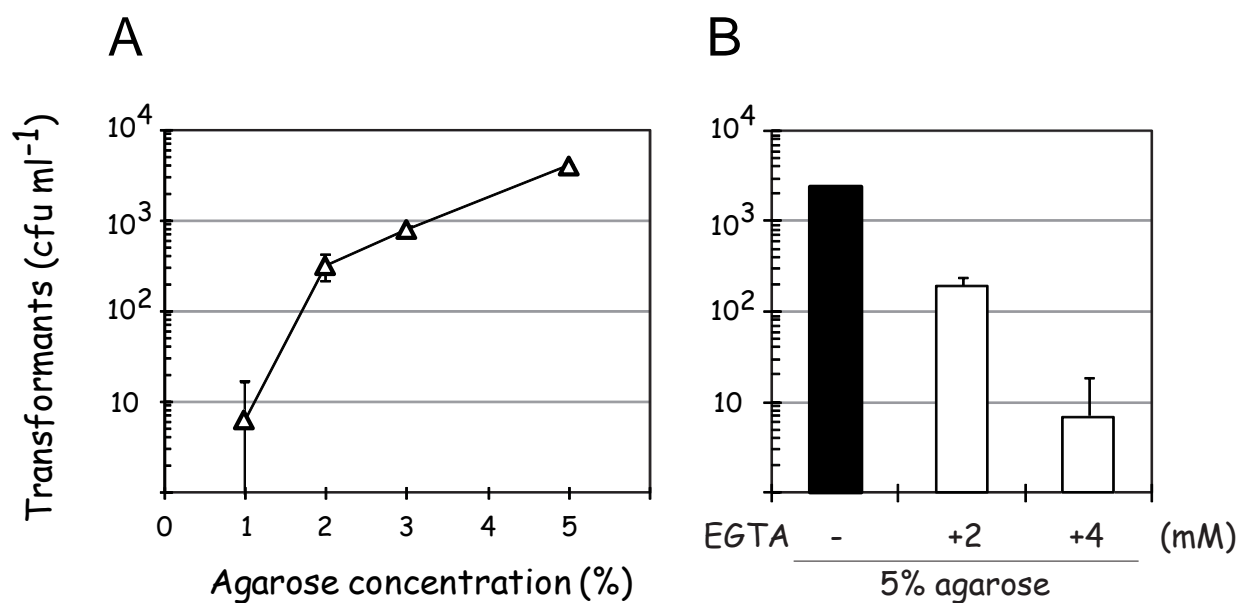
FIG. S2. Effect of agarose concentration and of EGTA on plasmid transformation efficiency. 50 μl of a ZK126 culture that had been incubated under static conditions were mixed with pDsRED plasmid DNA (Materials and Methods) and (A) immediately spread on selective plates containing different agarose concentrations (Biowest Agarose, Gene Tech Company Ltd, Shanghai; gel strength 750 g/cm^2). (B) 0, 2 or 4 mM EGTA was added to the plates, as indicated respectively by -, +2 and +4.
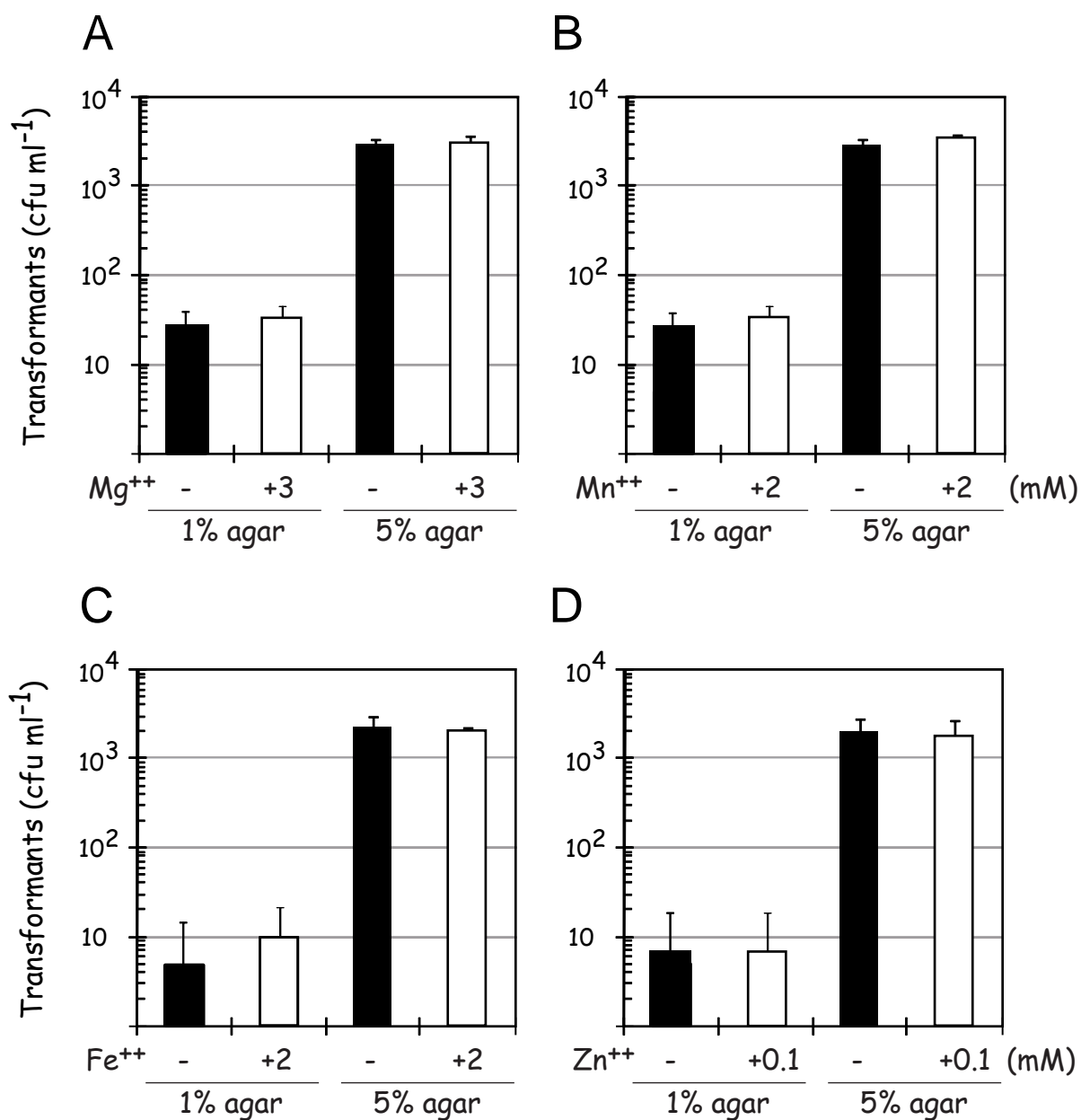
FIG. S3. Effect of Mg⁺⁺ (A), Mn⁺⁺ (B), Fe⁺⁺ (C), and Zn⁺⁺ (D) on plasmid transformation efficiency. 50 µl of a ZK126 culture that had been incubated under static conditions were mixed with pDsRED plasmid DNA (Materials and Methods) and immediately spread on selective plates containing the indicated % agar (Bacto^{TM} Agar). 0 or 3 mM Mg⁺⁺ (2 mM for Mn⁺⁺ and Fe⁺⁺, and 0.1 mM for Zn⁺⁺; the latter concentration was the highest not affecting growth under our experimental conditions) was added to the plates as indicated respectively by – and +.

Supplemental references

1. **Baba, T., T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Wanner, and H. Mori**. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2**:2006.

2. **Cameron, A. D. and R. J. Redfield**. 2006. Non-canonical CRP sites control competence regulons in *Escherichia coli* and many other gamma-proteobacteria. Nucleic Acids Res. **34**:6001-6014.

3. **Chen, I. and D. Dubnau**. 2004. DNA uptake during bacterial transformation. Nat. Rev. Microbiol. **2**:241-249.

4. **Claverys, J. P. and B. Martin**. 2003. Bacterial "competence" genes: signatures of active transformation, or only remnants? Trends Microbiol. **11**:161-165.

5. **Clifton, S. W., D. McCarthy, and B. A. Roe**. 1994. Sequence of the *rec-2* locus of *Haemophilus influenzae*: Homologies to *comE*-ORF3 of *Bacillus subtilis* and *msbA* of *Escherichia coli*. Gene **146**:95-100.

6. **Datsenko, K. A. and B. L. Wanner**. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A **97**:6640-6645.

7. **Dougherty, B. A. and H. O. Smith**. 1999. Identification of *Haemophilus influenzae* Rd transformation genes using cassette mutagenesis. Microbiology **145**:401-409.

8. **Finkel, S. E. and R. Kolter**. 2001. DNA as a nutrient: novel role for bacterial competence gene homologs. J. Bacteriol. **183**:6288-6293.

9. **Karudapuram, S., X. Zhao, and G. J. Barcak**. 1995. DNA sequence and characterization of *Haemophilus influenzae dprA*$^+$, a gene required for chromosomal but not plasmid transformation. J. Bacteriol. **177**:3235-3240.

10. **Palchevskiy, V. and S. E. Finkel**. 2006. *Escherichia coli* competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient. J. Bacteriol. **188**:3902-3910.

11. **Redfield, R. J., A. D. Cameron, Q. Qian, J. Hinds, T. R. Ali, J. S. Kroll, and P. R. Langford**. 2005. A novel CRP-dependent regulon controls expression of competence genes in *Haemophilus influenzae*. J. Mol. Biol. **347**:735-747.

12. **Sauvonnet, N., P. Gounon, and A. P. Pugsley**. 2000. PpdD type IV pilin of *Escherichia coli* K-12 can Be assembled into pili in *Pseudomonas aeruginosa*. J. Bacteriol. **182**:848-854.

13. **Smeets, L. C., S. C. Becker, G. J. Barcak, C. M. Vandenbroucke-Grauls, W. Bitter, and N. Goosen**. 2006. Functional characterization of the competence protein DprA/Smf in *Escherichia coli*. FEMS Microbiol Lett. **263**:223-228.

14. **Sun, D., Y. Zhang, Y. Mei, H. Jiang, Z. Xie, H. Liu, X. Chen, and P. Shen**. 2006. *Escherichia coli* is naturally transformable in a novel transformation system. FEMS Microbiol Lett. **265**:249-255.

15. **Tomb, J.-F., G. J. Barcak, M. S. Chandler, R. J. Redfield, and H. O. Smith**. 1989. Transposon mutagenesis, characterization, and cloning of transformation genes of *Haemophilus influenzae* Rd. J. Bacteriol. **171**:3796-3802.

16. **Tomb, J.-F., H. El-Hajj, and H. O. Smith**. 1991. Nucleotide sequence of a cluster of genes involved in the transformation of *Haemophilus influenzae* Rd. Gene **104**:1-10.

17. **VanWagoner, T. M., P. W. Whitby, D. J. Morton, T. W. Seale, and T. L. Stull**. 2004. Characterization of three new competence-regulated operons in *Haemophilus influenzae*. J. Bacteriol. **186**:6409-6421.

Supplemental Materials 2

Aim of the Supplemental Experiments

Sxy and plasmid transformation of E. coli *on plates.*

Sxy regulates competence for genetic transformation in *H. influenzae*, *V. cholerae* and *A. pleuropneumoniae* by controlling the transcription of DNA uptake genes (1, 3, 5). It has also been proposed to control transcription of a putative competence regulon in *E. coli* (2, 6). As described in the reference (7), DNA uptake gene homologs are not required for plasmid transformation in *E. coli*. Interestingly, *sxy* inactivation was found to affect transformation of *E. coli* at a low Ca^{2+} concentrations (10 mM) in the liquid culture (6). However, the role of Sxy is unknown in natural plasmid transformation of *E. coli* on plates. To characterize a potential role of *sxy* in natural plasmid transformation *E. coli* on plates, I compared transformation frequency between a *sxy* mutant and its wildtype parent (BW25113).

Effects of filters on plasmid tranformation.

Agar/agarose concentration strongly affects spontaneous plasmid transformation frequency in *E. coli* (7). To account for this stimulatory effect, the size of the meshes which presumably depends on agar/concentration was proposed to affect the availability of DNA on the surface of LB-agar plates. I therefore investigated whether the placement of filters on the surface of LB agar plates would allow me to check this hypothesis.

Results and Discussion

Effect of sxy *inactivation on natural plasmid transformation of* E. coli *cells on solid medium*

The data in Table S1 showed that *sxy* inactivation did not have a significant effect on transformation of *E. coli* on a LB-agar plate which contains less than 2 mM of Ca^{2+}. The result is in good agreement with our previous report that transforming DNA uptake gene horthologs are not required for plasmid transformation on plate (7) and further

strengthens the conclusion that transforming DNA uptake gene homologs do not mediate plasmid transformation of *E. coli* cells on solid medium.

Table S1. Effect of filters on natural plasmid transformation on LB-agar plates *.

	WT	*Δsxy*
Experiment 1	2.95×10^{-6}	4.54×10^{-6}
Experiment 2	0.82×10^{-6}	0.985×10^{-6}
Experiment 3	1.474×10^{-6}	0.8×10^{-6}

* After static incubation in a beaker covered by an air permeable membrane at 37℃, pDsRED (2 μg) was mixed with the static culture (50 μl) thoroughly and the mixture was spread onto 5% LB-agar (Bacto Difco Agar) with ampicillin (100 μg/ml). Viable cells were counted by plating serial diluted mixture on 5% LB-agar without antibiotics. Data shown are transformation frequencies (the number of transformants on LB-agar with ampicillin versus the number of viable cells on LB-agar plates) in three independent experiments.

Effect of filters on natural plasmid transformation on LB-agar plates

Although agar/agarose can strongly stimulate spontaneous plasmid transformation in *E. coli* and the divalent cations chelator EGTA can inhibit transformation on LB-agar plates, no divalent cations contained in the agar/agarose have been screened out as a transformation stimulator yet (7). An alternative hypothesis is proposed to explain transformation stimulation by agar/agarose. The size of the meshes on the surface of LB-agar plates are determined by the concentration of agar. The mesh size in 1% agarose and 5% agarose plates were thought to be larger than 4 μm and smaller than 2 μm respectively (4). In natural plasmid transformation of *E. coli*, DNA on the surface of plates may be easily trapped into the meshes on plates with a low agar concentration (1%), which would cause barriers for DNA binding/uptake by the recipient cells. To know whether smaller meshes at the surface of high agar concentration plates were responsible for the observed high transformation efficiency, I placed filters (nylon filter

or nitrocellulose filter) with a pore size of 2 μm on the surfaces of LB-agar plates containing 1% and 5% agar. The addition of a nylon filter or a nitrocellulose filter on the surface of these LB-agar plates did not suppress cell growth on LB-agar containing either 1% or 5% agar (Table S1). However, the presence of a filter indeed reduced transformation significantly. These inhibitory effect of filters did not allow us to conclude as to whether pore size on the surface of LB-agar plates plays a role in transformation, as the filters may bring additional barriers (e.g. DNA may not be able to move on the filters as well as on LB-agar surface).

Table S2 Suppression of transformation by filters on LB-agar plates*.

Exp 1	5% agar	1% agar	1% agar +Nylon filter
	4640 ± 880 (cfu ml^{-1})	40 ± 40 (cfu ml^{-1})	12 ± 24 (cfu ml^{-1})
Exp 2	5% agar	1% agar	1% agar +Nitrocellulose
	2840 ± 880 (cfu ml^{-1})	0	0
Exp 3	5% agar	5% agar + Nylon filter	5% agar +Nitrocellulose
	14880 ± 1720 (cfu ml^{-1})	440 ± 160 (cfu ml^{-1})	200 ± 80 (cfu ml^{-1})

* After static incubation in a beaker covered by an air permeable membrane at 37℃, pDsRED (2 μg) was mixed with the static culture (50 μl) thoroughly and the mixture was spread onto 1% LB-agar and 5% LB-agar (Bacto Difco) with or without ampicillin, where a nylon or nitrocellulose filter with a pore size of 2 μm was placed on the surface of LB-agar.

References

1   **Bosse, J. T., S. Sinha, T. Schippers, J. S. Kroll, R. J. Redfield, and P. R. Langford.** 2009. Natural competence in strains of *Actinobacillus pleuropneumoniae*. FEMS Microbiol Lett **298:**124-30.

2   **Cameron, A. D., and R. J. Redfield.** 2006. Non-canonical CRP sites control competence regulons in *Escherichia coli* and many other gamma-proteobacteria. Nucleic Acids Res **34:**6001-14.

3   **Meibom, K. L., M. Blokesch, N. A. Dolganov, C. Y. Wu, and G. K. Schoolnik.**

2005. Chitin induces natural competence in *Vibrio cholerae*. Science **310:**1824-7.

4   **Pernodet, N., M. Maaloum, and B. Tinland.** 1997. Pore size of agarose gels by atomic force microscopy. Electrophoresis **18:**55-8.

5   **Redfield, R. J.** 1991. *sxy*-1, a *Haemophilus influenzae* mutation causing greatly enhanced spontaneous competence. J Bacteriol **173:**5612-8.

6   **Sinha, S., A. D. Cameron, and R. J. Redfield.** 2009. Sxy induces a CRP-S regulon in *Escherichia coli*. J Bacteriol **191:**5180-95.

7   **Sun, D., X. Zhang, L. Wang, M. Prudhomme, Z. Xie, B. Martin, and J. P. Claverys.** 2009. Transforming DNA uptake gene orthologs do not mediate spontaneous plasmid transformation in *Escherichia coli*. J Bacteriol **191:**713-9.

Chapter 3

Attempts at Further Documenting so-called Nutritional

Competence of *Escherichia coli*

The experimental work described below was entirely carried out in the Rosie REDFIELD laboratory (The University of British Columbia; Life Sciences Centre, Health Sciences Mall, UBC Point Grey campus, Vancouver, Canada) during a visit (Feb 1st-May 1st, 2010).

A previous independent attempt at reproducing FINKEL and coworkers' published data on *E. coli* nutritional competence had been made before at the LMGM in Toulouse; published observations could not be confirmed using strains kindly provided by Steven FINKEL.

Similar attempts at growing *E. coli* cells on DNA conducted in the Susan GOTTESMAN laboratory have also failed (personal communication).

Summary

Transforming DNA uptake gene homologs *hofQ*, *ppdD* and *ycaI* which potentially encode, respectively, outer membrane channel protein, part of the transformation pseudopilus and the inner membrane channel protein, do not mediate natural plasmid transformation of *Escherichia coli* on solid medium. On the other hand, *E. coli* was reported to display the ability to use DNA as the sole carbon source. This ability was called 'nutritional competence' and *hofQ* was shown to be required for this process. However, it remains unknown how exogenous DNA is translocated into the cytoplasm and how nutritional competence is regulated. To get insights into DNA translocation and nutritional competence regulation, I wished to investigate nutritional competence in *E. coli ycaI* and *sxy* mutants. The latter is the orthologue of the main regulator of competence in *Haemophilus influenzae*. I first attempted to repeat the well documented experiment for assaying the use of DNA as a nutrient using the wild type *E. coli* strain ZK126, which has been reported to be able to take up DNA as food. I found that cell growth at low density responded sensitively to a contaminant in glassware. By treating glassware with acid and washing it with distilled water, I prepared nutrient-free glassware. Nutrient-free glassware allowed me to observe cell growth in M63 minimal medium with glucose as carbon source and no growth without glucose. To my surprise, no effect of DNA on cell growth was observed, including in M63 minimal medium containing a low concentration of glucose to prevent cell death. As I was unable to reproduce nutritional competence experiments, the roles of *ycaI* and *sxy* in this process remain elusive. Together, my data do not support the use of DNA as a nutrient by *E. coli* cells. To validate whether *E. coli* is able to use DNA as a nutrient, it is necessary to seek independent confirmation.

Introduction

Natural transformation, defined as the phenomenon of taking up foreign DNA into a bacterium to acquire new traits, has been documented in more than 70 bacterial species distributed in far-related phyla, including the Firmicutes *Bacillus subtilis* and *Streptococcus pneumoniae*, and the proteobacterium *Haemophilus influenzae* (10, 11).

Natural transformation relies on a conserved DNA uptake machinery enabling extracellular DNA to pass through bacterial membranes (5, 8). The current DNA uptake model postulates an initial step, specific to Gram-negative bacteria, the translocation through the outer membrane, which involves a channel formed by an outer membrane (OM) porin (e.g. ComE in *H. influenzae*). Then, extracellular double stranded DNA (dsDNA) crosses the peptidoglycan barrier with the assistance of a pilus fiber (transformation pseudo-pilus), which extrudes and retracts to bring dsDNA in contact with a receptor known as ComEA in *B. subtilis* and *S. pneumoniae*. A nuclease, known in *S. pneumoniae* only as EndA, then degrades one strand allowing uptake with 3' to 5' polarity of its single-stranded DNA (ssDNA) complement through the membrane via a channel formed by ComEC (2, 5, 8, 12, 13).

While the DNA uptake machinery is

conserved among naturally transformable bacteria, it is not permanently present. Many transformable species develop a transient physiological state, termed competence, during which they express DNA uptake genes. Conditions for competence induction and the regulatory circuits are largely species specific (6, 15, 19). For example, shifting *H. influenzae* from nutrient rich medium to nutrient poor medium increases the expression of a transcriptional regulator Sxy and cAMP receptor CRP, which stimulate the transcription of genes for DNA uptake and processing (3, 4), whereas *S. pneumoniae* cells develop competence as a general response to environmental stress (7).

Although so far the proteobacterium *Escherichia coli* has not been considered a naturally transformable species, several independent groups reported that *E. coli* was transformable under 'natural' conditions (1, 14, 22, 23). In addition, *E. coli* was reported to be able to grow in a medium containing DNA as the sole carbon source, suggesting that this bacterium may possess the ability to take up exogenous DNA (9). On the other hand, bioinformatics (6) and transcriptome analyses (20) provide clues suggesting that *E. coli* could well belong to the naturally transformable species. *E. coli* has thus a complete set of genes potentially encoding orthologues of all proteins required for assembling the transforming DNA uptake machinery, as well as key regulators for turning on expression of this gene set (see Thesis Introduction).

To establish whether these competence gene homologs mediate DNA uptake in natural plasmid transformation of *E. coli* cells on plates (22), we examined transformation rates in mutants defective for the putative OM porin encoding gene *hofQ* or in the IM channel gene *ycaI*. Neither mutant displayed reduced plasmid

transformation, showing that the putative transforming-DNA-uptake machinery does not mediate spontaneous plasmid transformation in *E. coli* (21). We also investigated a *sxy* mutant but obtained no evidence that this putative competence regulation gene was important for plasmid transformation (see Thesis, Chapter 2 Supplemental Material).

During the study of nutritional competence, several genes were found to be required for the use of DNA as carbon source (17, 18). However, among the studied gene set, only *hofQ* was predicted to be important for DNA uptake as it encodes a putative OM porin (9, 17).

In this study, we planned to investigate whether nutritional DNA traverses across the IM through the putative channel protein YcaI. We also planned to establish whether the competence regulator Sxy was required for nutritional competence, which would provide a direct connection between the use of DNA as a nutrient by *E. coli* cells, the *com* regulon and natural competence induction in this species. Unfortunately, despite numerous attempts, we failed to observe any DNA-dependent growth of *E. coli* cells in M63 minimal medium, including using strains kindly provided by S. Finkel. These attempts are presented in the Results section. This manuscript is accompanied by a Supplemental Discussion which presents an in-depth analysis of the previous publications on nutritional competence (see Chapter 3, Supplemental Discussion).

Materials and Methods

Bacterial strains. All experiments were performed with *E. coli* strain ZK126 (W3110 *lacU169 tna-2*) and its derivatives. ZK126 was

kindly donated by Dr. Steven Finkel (9). Strains JPC1001 (ZK126::*hofQ*::*cat*) and JPC1004 (ZK126::*ycaI*::*cat*) were constructed in our previous work (21).

Media preparation. M63 minimal medium (13.6 g/L KH_2PO_4, 2 g/L $(NH_4)_2SO_4$, 0.5 mg/L $FeSO_4 \cdot 7H_2O$) and $MgSO_4$ (1 M) solution were prepared separately and sterilized by autoclave (16). VB1 (10 mg/ml) was sterilized by filtration. To prevent contamination by residual nutrients, the filter was washed by 50 ml of in-house distilled water, followed by 5 ml of distilled water (Invitrogen Company) before use for filter sterilization of VB1 solution. To prevent contamination during pH measurement, 3 ml of M63 medium were transferred to a disposable tube and pH was measured with a pH meter. The final pH of M63 was adjusted to 7.0 with KOH (5 M) before sterilization. Then, filter sterilized VB1 was added to the sterilized M63 medium. The final M63 medium contains 100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 1.8 μM $FeSO_4$ and 1 μg/ml VB1.

DNA preparation. Before sonication, the sonicator probe was cleaned to avoid potential contamination by washing with in-house distilled water several times. Then the probe was washed by ethanol and distilled water twice. Solid salmon sperm DNA, purchased from Sigma company, was dissolved in distilled water. The DNA was sonicated in a nutrient-free glass tube placed in ice, at a high power in pulsed modus (10 sec on, 10 sec off) for ~40 min to reduce its size to ~500 bp. The size of sonicated DNA fragments was examined by gel electrophoresis.

To get rid of potential protein contamination or other potential soluble nutrients, the sonicated DNA solution was mixed with phenol/chloroform in a volume ratio of 1:1 thoroughly and centrifuged to separate DNA solution and phenol/chloroform, followed by treatment with chloroform the same as phenol/chloroform described above. Ethyl ether was added to the extracted DNA solution to get rid of residual chloroform. DNA solution was extracted with phenol/chloroform, chloroform and ether three rounds (9). Then, the DNA was precipitated by ethanol with NaCl (3 M), washed by 70% EtOH and air dried at 37°C. The final DNA concentration was measured with a Nanodrop spectrophotometer.

Culture conditions. To get rid of residual nutrients in the inoculum, cells grown overnight in Lubria-Bertani broth (~ 5×10^9 CFU/ml) were collected by centrifugation. The supernant was discarded and cell pellets were washed with M63 and then diluted to 1: 5,000,000 (~ 1000 CFU/ml). Immediately before use, sonicated ultrapure salmon sperm DNA solution was again precipitated with ethanol. Then cells in M63 plus 1 mg/ml of freshly precipitated salmon sperm DNA were incubated in a roller at 37 ℃. Cell titers were measured periodically. DNA concentration in the cell culture was measured with a Nanodrop.

To determine cell growth in the minimal medium with a low concentration of glucose as carbon source, glucose was supplemented to the M63 at final concentrations of 0.000001％, 0.00001%, 0.0001%, 0.001%, 0.01%, 0.1% in M63.

Results and Discussion

Attempt at reproducing DNA-dependent growth experiments with *E. coli* ZK126 cells. In order to check the possible involvement of *ycaI* or *sxy* in nutritional competence, we first tried to reproduce the previously published growth experiments of *E. coli* cells in chemically-defined M63 minimal medium supplemented with 1 mg/ml salmon sperm DNA as the sole carbon source, in glass tubes. Wild type *E. coli* ZK126 and its two derivatives *hofQ* (strain JPC1001) and *ycaI* (strain JPC1004) mutants (Materials and Methods) were used. Quite surprisingly, for all three stains, the growth pattern and final growth yield were

almost identical (Fig. 1). Over 5-day incubation, cell density increased from 10^3 CFU/ml to more than 10^5 CFU/ml (Fig. 1).

Even more surprising, growth was also observed with all three strains in M63 minimal medium but without any added carbon source (Fig. 1). After 5 days of incubation, cell density increased from 10^3 CFU/ml to more than 10^4 CFU/ml (Fig. 1).
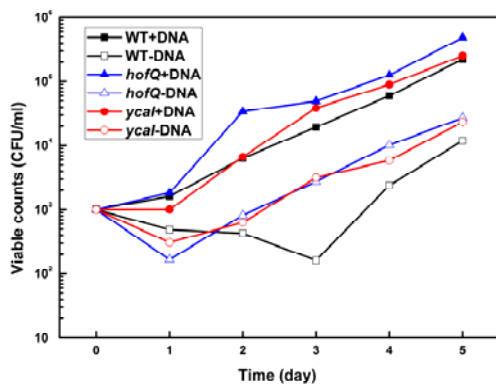

Fig. 1. Growth of *E. coli* in 'standard' glass tubes. ZK126 (black rectangle) and its *hofQ* (blue triangle) and *ycaI* (red circle) mutant derivatives were grown overnight in LB broth, diluted to 1: 5,000,000 in M63 and inoculated into M63 supplemented with (filled symbols) or without (open symbols) 1 mg/ml of salmon DNA (final cell concentration is ~1,000 CFU/ml) in non acid-treated glass tubes ('standard' glass tubes). Cell densities on day 0 were normalized to 1,000 CFU/ml, and cell densities in the next days were calculated using day 0 normalization as reference. During incubation in a rotator at 37 ℃, cell titers were measured every day over a 5 day period.

While the observation of a 10-fold higher cell density in DNA-containing cultures compared to cultures without DNA could indicate that DNA was used by *E. coli* cells, the finding that *hofQ* mutant cells behaved similarly to wildtype cells appeared puzzling since *hofQ* was previously concluded to be required for the use of DNA (9). In addition, the observation that *E. coli* cells could grow

in minimal medium without any carbon source strongly suggested the presence of residual nutrients in the containers (e.g. glass tubes). These observations prompted us to extensively wash glassware and use ultra-clean glassware (referred to as 'nutrient-free' hereafter) to perform the next experiments.

Preparation of nutrient-free glassware and validation for growth in M63 minimal medium. Glassware was first filled with
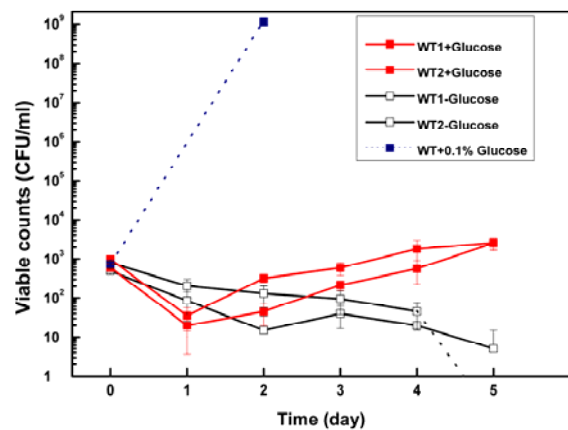

Fig. 2. Cell growth in nutrient-free glass tubes. Overnight grown *E. coli* ZK126 in LB broth was inoculated 1:5,000,000 (vol/vol) into M63 supplemented with 0.1% (blue filled rectangle, dash line), 0.000001% glucose (red filled rectangle, solid line) or no glucose (black open rectangle, solid line). The culture was incubated at 37℃ and cell titers were measured daily. The black dash line indicates that the cell titer is below the detection limit. Data shown are means of duplicate samples. Error bars indicate standard deviation.

0.5 M HCl and kept overnight at room temperature, followed by washing with in-house distilled water trice and rinsing with distilled water from company. All the following experiments were performed with acid-treated and extensively washed glassware. Before assaying DNA-dependent growth in nutrient-free glass tubes, we first
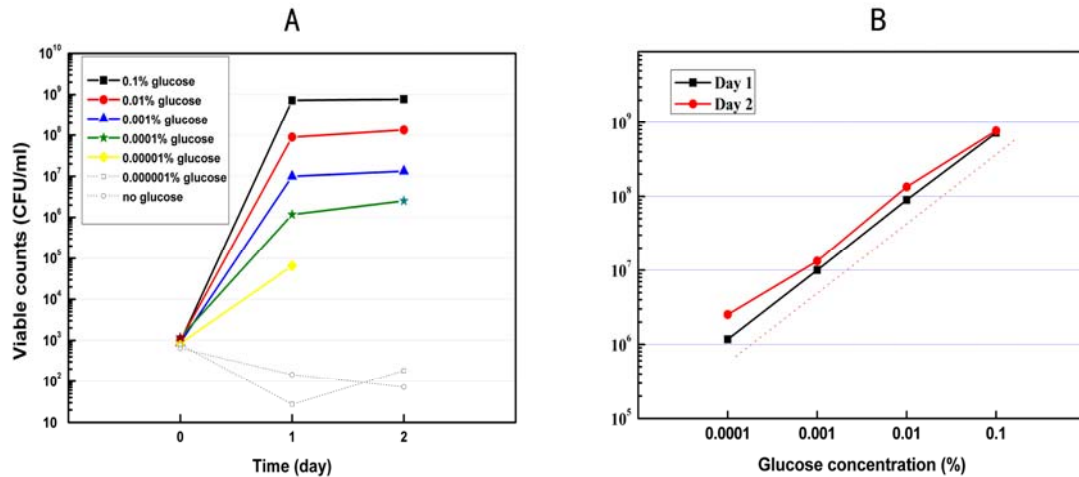
Fig. 3. Effect of glucose concentration on cell growth in nutrient-free glass tubes. (A) *E. coli* ZK126 cells grown overnight in LB broth was inoculated at 1:5,000,000 (vol/vol) dilution into M63 medium supplemented with 0.1% (black filled rectangle in panel A), 0.01% (red filled circle in panel A), 0.001% (blue filled triangle in panel A), 0.0001% (navy filled pentacle in panel A), 0.00001% (yellow filled rhombus in panel A), 0.000001% glucose (black open rectangle, grey line) or no glucose (black open circle, grey line). Cell titers were measured 0, 1 and 2 days after incubation at 37°C. (B) Growth yield expressed as a function of glucose concentration. Black curve and symbols, growth yield after one day incubation; red curve and symbols, growth yield after 2 days incubation. The orange dash line indicates slope of 1 in the panel B. Data are means of duplicate samples.
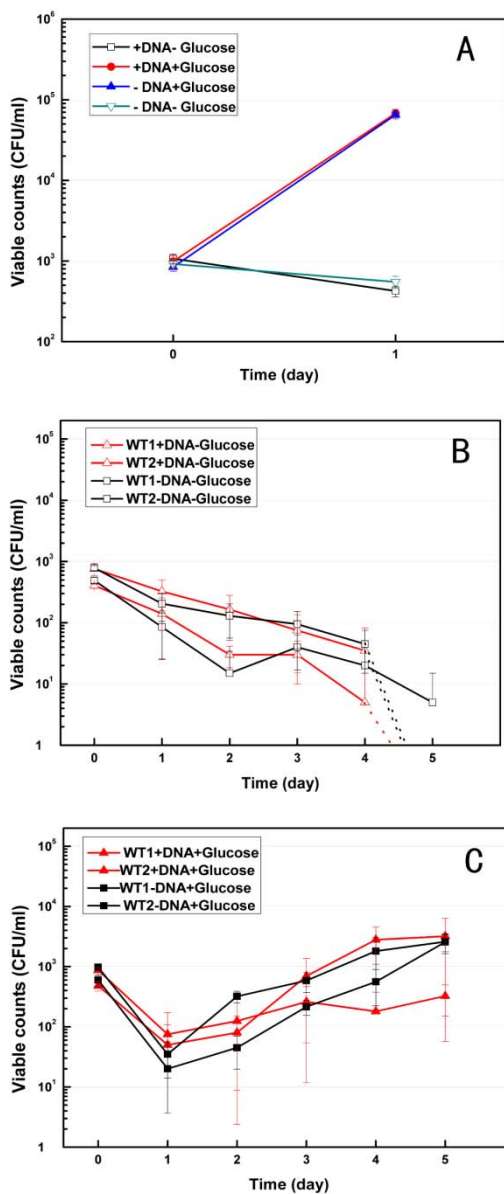
examined the ability of *E. coli* ZK126 to grow in M63 supplemented with glucose.

In contrast to growth experiments in glass tubes, we found that *E. coli* ZK126 could not grow in 'nutrient-free' glassware in the absence of glucose. A decrease in the number of viable cell counts was even observed in the absence of glucose; after 5 days incubation, cell density was close to 0 (Fig. 2). Cell death was more or less prevented in the presence of 0.000001% glucose, while 0.1% glucose allowed *E. coli* cells to reach a density of more than 10^9 CFU/ml within 2 days, indicating that M63 minimal medium supplemented with this concentration of glucose fully supported growth (Fig. 2).

Nutrient-free glassware allows growth with limiting glucose concentrations. Growth of ZK126 was then examined in nutrient-free glassware containing M63 minimal medium supplemented with various limiting amounts of glucose.

Whatever the concentration of glucose, *E. coli* reached the maximal growth within 1 day (Fig. 3A). Final growth yields after 2 days incubation were identical to those after 1 day and were dependent on glucose concentration (Fig. 3B).

We concluded from the observed direct relationship between glucose concentration and final growth yield that carbon source represented the main limitation for growth of *E. coli* cells in M63 minimal medium in nutrient-free glassware.

Inability to use DNA as a carbon source for growth in M63 in nutrient-free glassware. It has been reported that *E. coli* cells were able to grow in M63 with 0.1% DNA as the sole carbon source (17). A cell-density increase of ~120-fold was observed for the wild type after only 24-h incubation (see Chapter 3 Supplemental Discussion). We therefore investigated *E. coli* cell growth in M63 minimal medium with salmon sperm

Figure 4. Investigating the effect of salmon sperm DNA on cell growth in nutrient-free glass tubes. (A) *E. coli* ZK126 grown overnight in LB broth were inoculated 1: 5,000,000 (vol/vol) into M63 supplemented with both 0.00001% glucose and 1 mg/ml of salmon sperm DNA (red filled circle); with only 0.00001% glucose (blue filled triangle); with only 1 mg/ml of salmon sperm DNA (black open rectangle); or with neither glucose nor DNA (cyan open triangle). (B) Panel B and C: Diluted *E. coli* ZK126 culture was inoculated into M63 supplemented with or without 1 mg/ml of salmon sperm DNA in the presence of 0.000001% glucose (Panel B) or no glucose (Panel C). Red open triangle: with DNA but without glucose; black open rectangle: with neither DNA nor glucose; red filled triangle: with both DNA and glucose; black filled rectangle: without DNA but with glucose. Data shown are means of duplicate samples. Error bars indicate standard deviation.

DNA as the sole carbon source in nutrient-free glassware. Firstly, we examined the effect of DNA on cell growth with or without 0.00001% glucose as the carbon source. The addition of 1 mg/ml of salmon sperm DNA had no detectable effect on cell growth either in the presence of a low concentration of glucose or in the absence of glucose after one day of incubation (Fig 4A).

We then examined the use of DNA for growth in M63 minimal medium in the absence of glucose over an extended limited amount of glucose (0.00000%). Again added DNA had no effect on growth or survival of *E. coli* cells over 5 days incubation (Fig. 4C). Monitoring DNA concentration in the cultures (Materials and Methods) revealed that DNA concentration remained constant during incubation (Data not shown).

Taken together, our data do not support previous reports that *E. coli* cells are able to use DNA as carbon source. In light of the variable growth yield that we observed when using non HCl-treated glassware (Fig. 1 and data not shown), we suspect that glassware contamination could provide residual nutrients that interfere with the outcome of growth experiments.

Concluding Remarks

Since we failed to observe

DNA-dependent growth in *E. coli*, we were unable to determine whether *ycaI* and *sxy*, which respectively encode the putative IM channel for DNA entry and the putative main regulator of competence, are required for the use of DNA as a carbon source. An independent confirmation of the previously published DNA-dependent growth experiments is necessary to establish whether *E. coli* is able to use DNA as a nutrient.

References

1   **Baur, B., K. Hanselmann, W. Schlimme, and B. Jenni.** 1996. Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. Appl Environ Microbiol **62:**3673-8.

2   **Bergé, M., M. Moscoso, M. Prudhomme, B. Martin, and J. P. Claverys.** 2002. Uptake of transforming DNA in Gram-positive bacteria: a view from *Streptococcus pneumoniae*. Mol Microbiol **45:**411-21.

3   **Cameron, A. D., and R. J. Redfield.** 2008. CRP binding and transcription activation at CRP-S sites. J Mol Biol **383:**313-23.

4   **Cameron, A. D., and R. J. Redfield.** 2006. Non-canonical CRP sites control competence regulons in *Escherichia coli* and many other gamma-proteobacteria. Nucleic Acids Res **34:**6001-14.

5   **Chen, I., and D. Dubnau.** 2004. DNA uptake during bacterial transformation. Nat Rev Microbiol **2:**241-9.

6   **Claverys, J. P., and B. Martin.** 2003. Bacterial "competence" genes: signatures of active transformation, or only remnants? Trends Microbiol **11:**161-5.

7   **Claverys, J. P., M. Prudhomme, and B. Martin.** 2006. Induction of competence regulons as a general response to stress in gram-positive bacteria. Annu Rev Microbiol **60:**451-75.

8   **Dubnau, D.** 1999. DNA uptake in bacteria. Annu Rev Microbiol **53:**217-44.

9   **Finkel, S. E., and R. Kolter.** 2001. DNA as a nutrient: novel role for bacterial competence gene homologs. J Bacteriol **183:**6288-93.

10   **Johnsborg, O., V. Eldholm, and L. S. Havarstein.** 2007. Natural genetic transformation: prevalence, mechanisms and function. Res Microbiol **158:**767-78.

11   **Lorenz, M. G., and W. Wackernagel.** 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev **58:**563-602.

12   **Méjean, V., and J. P. Claverys.** 1993. DNA processing during entry in transformation of *Streptococcus pneumoniae*. J Biol Chem **268:**5594-9.

13   **Méjean, V., and J. P. Claverys.** 1988. Polarity of DNA entry in transformation of *Streptococcus pneumoniae*. Mol Gen Genet **213:**444-8.

14   **Maeda, S., A. Sawamura, and A. Matsuda.** 2004. Transformation of colonial *Escherichia coli* on solid media. FEMS Microbiol Lett **236:**61-4.

15   **Meibom, K. L., M. Blokesch, N. A. Dolganov, C. Y. Wu, and G. K. Schoolnik.** 2005. Chitin induces natural competence in *Vibrio cholerae*. Science **310:**1824-7.

16   **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

17   **Palchevskiy, V., and S. E. Finkel.** 2006. *Escherichia coli* competence gene homologs are essential for competitive

fitness and the use of DNA as a nutrient. J Bacteriol **188:**3902-10.

18   **Palchevskiy, V., and S. E. Finkel.** 2009. A role for single-stranded exonucleases in the use of DNA as a nutrient. J Bacteriol **191:**3712-6.

19   **Redfield, R. J., A. D. Cameron, Q. Qian, J. Hinds, T. R. Ali, J. S. Kroll, and P. R. Langford.** 2005. A novel CRP-dependent regulon controls expression of competence genes in *Haemophilus influenzae*. J Mol Biol **347:**735-47.

20   **Sinha, S., A. D. Cameron, and R. J. Redfield.** 2009. Sxy induces a CRP-S regulon in *Escherichia coli*. J Bacteriol **191:**5180-95.

21   **Sun, D., X. Zhang, L. Wang, M. Prudhomme, Z. Xie, B. Martin, and J. P. Claverys.** 2009. Transforming DNA uptake gene orthologs do not mediate spontaneous plasmid transformation in *Escherichia coli*. J Bacteriol **191:**713-9.

22   **Sun, D., Y. Zhang, Y. Mei, H. Jiang, Z. Xie, H. Liu, X. Chen, and P. Shen.** 2006. *Escherichia coli* is naturally transformable in a novel transformation system. FEMS Microbiol Lett **265:**249-55.

23   **Tsen, S. D., S. S. Fang, M. J. Chen, J. Y. Chien, C. C. Lee, and D. H. Tsen.** 2002. Natural plasmid transformation in *Escherichia coli*. J Biomed Sci **9:**246-52.

Chapter. 3 Supplemental Discussion: 'Nutritional Competence' of *Escherichia coli*: DNA-Uptake Gene Homologs and the Use of DNA as a Nutrient

Dongchang SUN   and Jean-Pierre CLAVERYS

Hereafter, we discuss three papers published by Finkel and coworkers on so-called nutritional competence of Escherichia coli*: (Finkel and Kolter, 2001), (Palchevskiy and Finkel, 2006) and (Palchevskiy and Finkel, 2009).*
To facilitate reading, relevant Figures from these papers have been reproduced and inserted in the body of the Discussion.

Comparing the ability of *E. coli* cells to use intact DNA versus DNA degradation products

Examination of Finkel and Kolter's data suggests that *E. coli* cells use DNA degradation products as a nutrient more efficiently than intact DNA. Thus after 4 days incubation in minimal medium which contained only inorganic salts, vitamin B1, and purified *E. coli* chromosomal DNA (added at a concentration of ~6 µg ml^{-1}) as the sole source of carbon, wild type cells were reported to reach a final density of 2 to 5 x 10^5 cfu ml^{-1} (Finkel and Kolter, 2001). However, when minimal medium containing added DNA was first pretreated with DNase I, wild-type cells reached a final cell density of ~2 x 10^9 cfu ml^{-1} (Finkel and Kolter, 2001), **suggesting that *E. coli* cells have a strong preference for DNA degradation products compared to intact DNA**.

Quantitative considerations on growth yields

Overall growth yields. Further examination of the reported experimental conditions from a quantitative point of view raises some intriguing questions. 6 µg ml^{-1} *E. coli* chromosomal DNA (i.e. the concentration added in the above mentioned experiment) corresponds to the chromosomal DNA content of ~10^9 cells assuming the presence of a single 6 Mb genome per cell (an underestimate as it is well known that, depending on growth rate, *E. coli* cells can engage in multiple chromosome replication cycles). As DNA constituted the only source of carbon, **this DNA concentration could by no mean permit the synthesis of twice this number of genomes *plus* all other cellular components** required to achieve the reported final cell density of ~2 x 10^9 cfu ml^{-1}. Therefore, the DNA concentration used in this experiment was clearly too low to reach this final cell density. The very same quantitative considerations also imply that the concentration of *E. coli* chromosomal DNA pre-treated with DNase I must have been much higher than 6 µg ml^{-1}.

Comparing growth yield with DNase I-digested DNA and with dNMPs. The final cell density reported with DNase I-digested DNA is also surprisingly high compared to the ~10^6 cfu ml^{-1} reached with 20 mM of either of the four dNMPs (except for TMP where cultures reached a density of 10^7 to 10^8 by day 2) (Palchevskiy and Finkel, 2006). It is striking that only nucleosides (at a concentration of 20 mM) could lead to cell densities close to 10^9 cfu ml^{-1}, whereas cells grown in dNMPs reached only ~10^6 cfu ml^{-1}. As DNase I produces nucleotides not nucleosides, these data are somehow difficult to reconcile.

Overall efficiency of 'nutritional competence'. In any case, final cell densities achieved with 1 mg ml^{-1} intact DNA remained ~20,000-fold lower than those reported with DNase I-digested DNA, clearly indicating that so-called **nutritional competence is a rather inefficient means of providing nutrients**. Taking this into account, one should avoid calling the underlying phenomenon 'nutritional competence'. On the other hand, even limited uptake of intact DNA could be important for cell survival during starvation conditions. For example, temporary repletion of nucleotide pools could allow the repair of damaged chromosomes. If this turned out to be the case, it would be more appropriate to call this phenomenon 'salvage DNA uptake' than 'nutritional competence'.

Variability in growth kinetics

It was first reported that in M63 minimal medium containing ~6 µg ml^{-1} sonicated *E. coli* chromosomal DNA as the sole source of carbon, wildtype cells exhibited a growth yield more than 50-fold greater than those of *yhiR** mutant cells **after 4 days incubation** [Fig. 3 in (Finkel and Kolter, 2001)].

**yhiR* was the first mutant isolated as 'stationary-phase-specific competition-defective' (Finkel and Kolter, 2001) (see below).
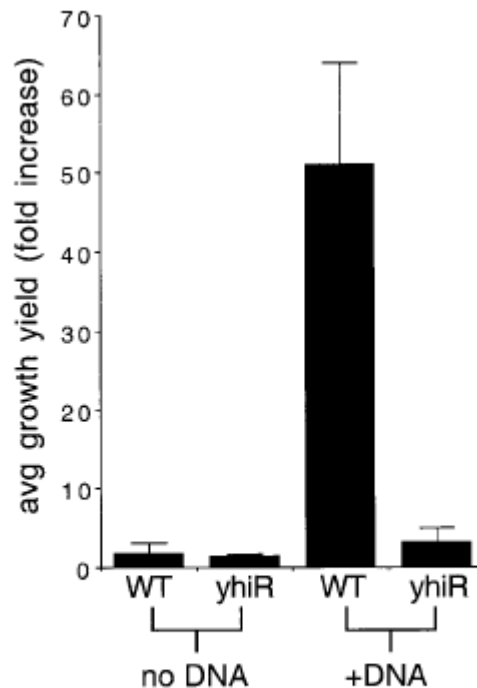
Reproduced from
(Finkel and Kolter, 2001)

FIG. 3. Growth of *E. coli* utilizing DNA as the sole carbon source. Cells were grown in M63 minimal medium in the presence or absence of purified *E. coli* chromosomal DNA as the sole carbon source. Results are averages of three experiments. WT, wild type.

In a second series of experiments, chromosomal *E. coli* DNA was substituted with 1 mg ml^{-1} (0.1%) ultrapure sonicated salmon sperm DNA (Palchevskiy and Finkel, 2006). This concentration increase did not change final growth yields resulting in cell-density increase of ~120-fold for the wild type compared to ≤3 fold for the mutants **but after only 24 h incubation** [Fig. 3 in (Palchevskiy and Finkel, 2006)].
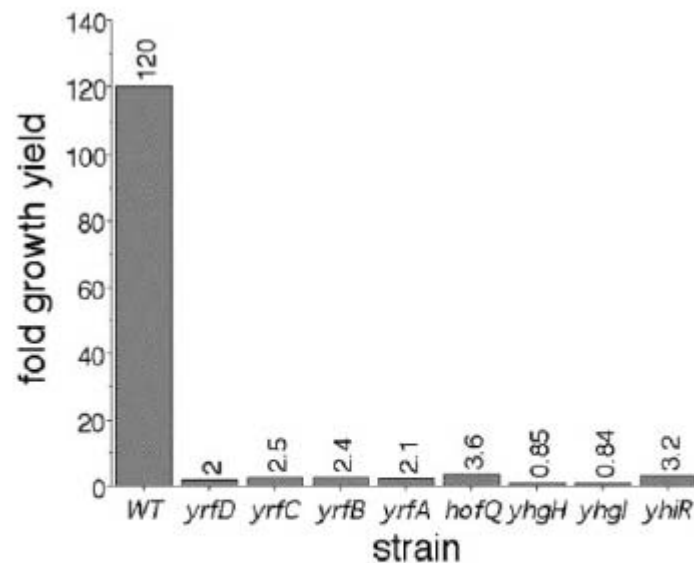
FIG. 3. Average growth yields of wild-type (WT) or *com* mutant cells in minimal medium supplemented with 0.1% ultrapure sonicated salmon sperm DNA as the sole source of carbon and energy. Growth yields (indicated above each bar) were determined by dividing the number of cells after 24 h of incubation by the number of cells at inoculation.

It is unclear whether the large increase in DNA concentration in (Palchevskiy and Finkel, 2006) accounts for the shorter incubation time as compared to previous work (Finkel and Kolter, 2001). Possibly, a similar increase in *E. coli* chromosomal DNA concentration would have given a similar final growth yield but with a shorter incubation time.

Theoretical considerations on the internalization of DNA via the DNA uptake machinery of naturally transformable bacteria

DNA concentration. It is of note that **1 mg ml^{-1} of DNA,** as used in (Palchevskiy and Finkel, 2006), **represents an enormous concentration** compared with the known saturating concentration for uptake of exogenous dsDNA by naturally transformable species such as *Bacillus subtilis* and *Streptococcus pneumoniae*, i.e. ~1 µg ml^{-1} chromosomal DNA (Barany and Tomasz, 1980).

Intactness of DNA provided as carbon source. The ability of *E. coli* cells to use DNA as a nutrient source was studied with sonicated dsDNA (0.5-2 kb long) (Finkel and Kolter, 2001), (Palchevskiy and Finkel, 2006). **The rationale for using sonicated DNA if it is to be taken up by the 'natural transformation machinery' is questionable** taking into account that during natural transformation of *Bacillus subtilis*, it has been shown that the mass of DNA bound at saturation is proportional to the molecular weight of the donor DNA molecules (Dubnau, 1991). Thus, if DNA binding is limiting as observed with naturally transformable bacteria, sonication might reduce the amount of DNA internalized by *E. coli* cells. It would therefore have been interesting to check whether longer (*i.e.*, non sonicated) DNA is more efficiently used by *E. coli* cells.

General considerations. The low efficiency of the DNA uptake process with respect to growth yield of *E. coli* cells gives credit to the view of Dubnau who pointed out that secretion of a nuclease into the medium and uptake of the nucleolytic products would seem to provide an efficient route for the consumption of environmental nucleic acids (Dubnau, 1999). Interestingly, recent observations with the naturally transformable species *Vibrio cholerae* provides direct support to this model. Strikingly enough, while nuclease production by low-density *V. cholerae* populations may foster rapid growth by providing a source of nucleotides for the repletion of nucleotide pools, static high-density populations stop nuclease production when competence develops, presumably to favor the uptake of intact DNA (Blokesch and Schoolnik, 2008).

Competitive fitness during long-term stationary-phase incubation and the use of DNA as a nutrient

In their initial experiment, Finkel and Kolter (2001) performed a genetic screen for 'stationary-phase-specific competition-defective' mutants. These mutants were selected from a transposon insertion library after 5 days coculture with wildtype cells (ZK1142) in Luria-Bertani (LB) broth. The first mutant isolated, *yhiR*, was outcompeted after 2 days coincubation in stationary phase and completely lost from the culture after 10 to 12 days [Fig. 1A in (Finkel and Kolter, 2001)]. Interestingly, *yhiR* mutant cells were reported to show a survival pattern identical to the wild type during long-term stationary phase incubation when grown separately from wild type cells [Fig. 1B in (Finkel and Kolter, 2001)].

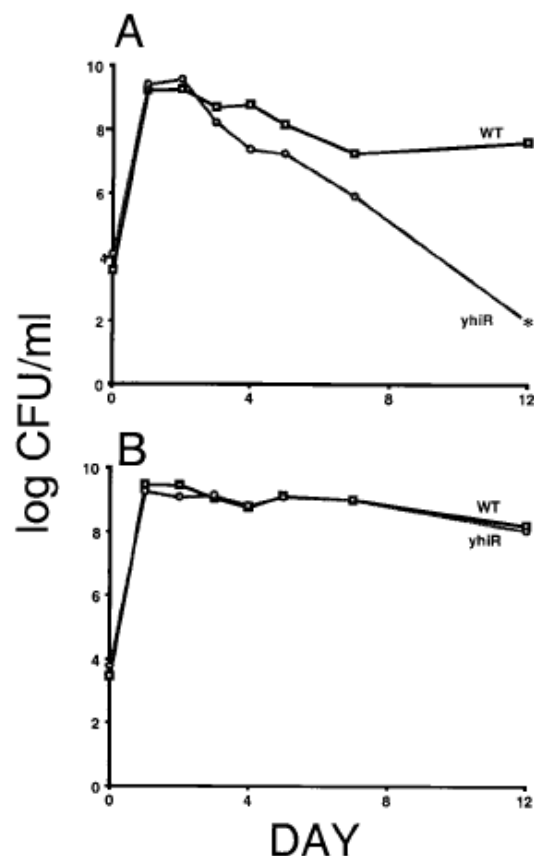Reproduced from
(Finkel and Kolter, 2001)

FIG. 1. Survival patterns of *yhiR* and the wild-type (WT) parental strain in the presence or absence of competition. LB cultures were incubated for 12 days. (A) Cells grown in coculture; (B) cells grown separately. The asterisk indicates no detectable counts (limit of detection of <100 CFU/ml).

hofQ mutant cells were reported to display a similar stationary-phase competition defect during coculture with the wild type with a complete loss from the culture after 12 days [Fig. 5 in (Finkel and Kolter, 2001)].
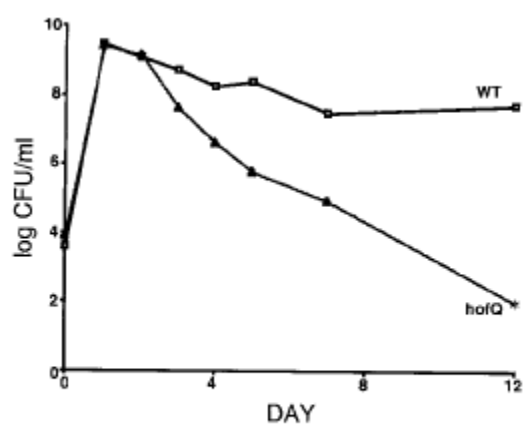
FIG. 5. Survival patterns of the *hofQ* mutant and the wild-type parental strain (WT) during competition in stationary phase. LB cultures were incubated for 12 days.

The co-cultivation loss was tentatively attributed to the inability of the *yhiR* mutant to catabolize double stranded DNA (but not DNA breakdown products) resulting in a competitive disadvantage during coculture with its wild-type parent. However, this explanation is not as straightforward as it looks at first sight. In particular, **it is not in itself sufficient to account for the fact that the death of *yhiR* cells occurs specifically during prolonged cocultures with wild-type cells but not when *yhiR* cells are incubated alone**. This death cannot be explained only by the consumption by wild-type cells of DNA released from dead cells, as this should not be detrimental to *yhiR* mutant cells which, in any case, are unable to use (released) DNA irrespective of the presence of wild-type competitors. This phenomenon rather suggests that wild-type cells are responsible for the exhaustion of some component(s) essential for long-term survival or for the production of toxic compounds. Even if this interpretation is correct, it remains difficult to understand why a similar exhaustion does not occur during long-term incubation of *yhiR* mutant cells alone or why *yhiR* mutant cells do not also produce the same toxic compounds.

Alternatively, wild-type cells could be responsible for the direct killing of *yhiR* mutant cells. This would not be unprecedented in view of the reported cannibalism in *B. subtilis* and fratricide in *S. pneumoniae* (Claverys and Håvarstein, 2007). However, the connection between such a putative killing capacity and the targeting of *yhiR* mutant cells only because they are unable to take up intact DNA would be unclear.

Variations in the degree of decrease of competitive fitness

In the first report, a complete loss of *yhiR* and *hofQ* cells from coculture in LB broth was reported to occur after 10 to 12 days (Finkel and Kolter, 2001). Intriguingly enough however, a less than 2-log reduction in the number of *yhiR* or *hofQ* cells compared to wild-type cells was reported in a more recent publication [Fig. 1B in (Palchevskiy and Finkel, 2006)]. In the latter publication, even after 20 days, the number of surviving mutant cells ranged between 10^4 and 10^5 cfu ml^{-1}. These variations are puzzling and possibly indicate that medium composition varies and can greatly affect the outcome of such competition experiments.
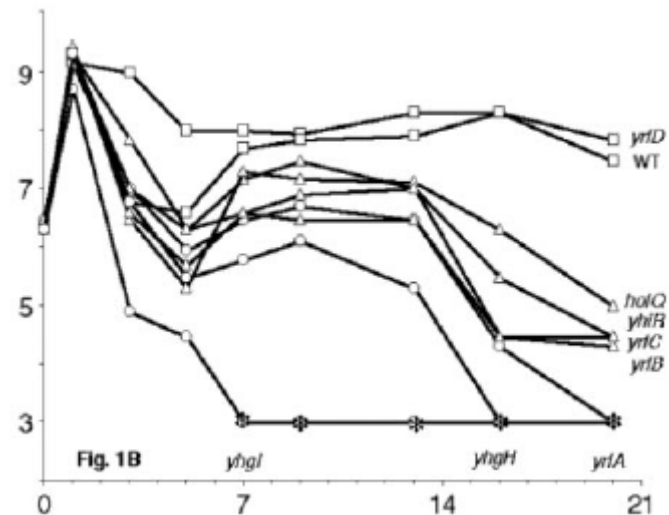
Ability to use dsDNA as a nutrient and competitive fitness

The relationship between the loss of the ability to use dsDNA as a nutrient and the loss of competitive fitness of mutants during long-term stationary-phase coculture with wild-type cells hold true for all mutants tested, with the remarkable exception of the *yrfD* (*hofM*) mutant (Palchevskiy and Finkel, 2006). **This mutant appeared unable to utilize salmon sperm DNA as sole source of carbon and energy**, showing an increase in cell density of 2-fold after 24 h incubation in M63 minimal medium, compared to a ~120-fold increase for wild-type cells [Fig. 3 in (Palchevskiy and Finkel, 2006); see above].

Intriguingly enough however, *yrfD* **mutant cells were not outcompeted by wild-type cells** even after 21 days incubation in LB broth   [Fig. 1B in (Palchevskiy and Finkel, 2006); see above]. In addition, when the relative fitness of this mutant was compared with others through simultaneous competition in a single culture of the eight mutant strains studied, *yrfD* mutant cells were present at the end of each experiment carried out [Fig. 2B in (Palchevskiy and Finkel, 2006)]. Thus, **the inability to use DNA as a nutrient does not systematically correlate with a loss of competitive fitness during long-term stationary-phase incubation**.
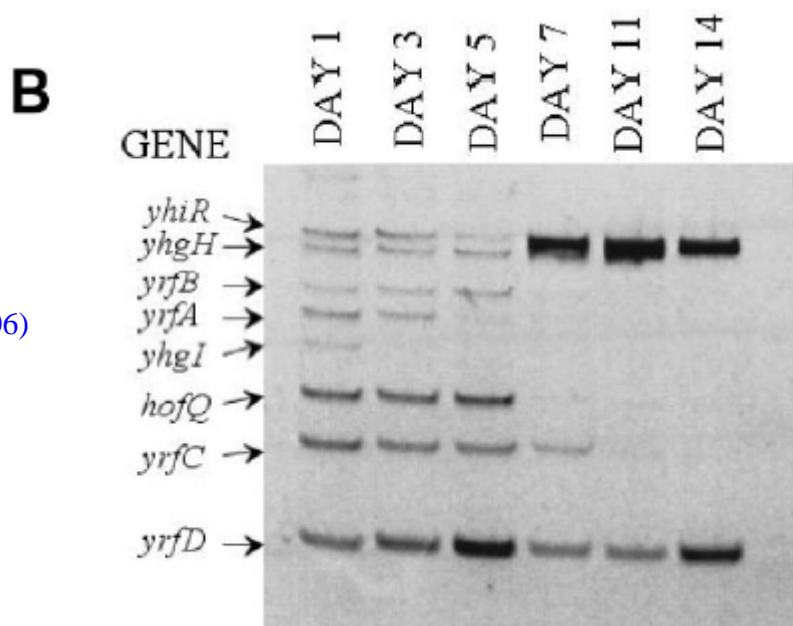
Uptake of DNA in *E. coli* cells via a machinery conserved in naturally transformable species?

Although *E. coli* was not known to be naturally competent for genetic transformation, the finding that homologs of *Haemophilus influenzae* and *Neisseria gonorrhoeae* competence genes were required for the use of DNA as a nutrient (Finkel and Kolter, 2001) led to the hypothesis that *E. coli* cells can develop 'nutritional competence' (Palchevskiy and Finkel, 2006). A working model of dsDNA uptake and metabolism based on models build for the well-known transformable species *B. subtilis* and *H. influenzae* was proposed (Palchevskiy and Finkel, 2006). According to this model, the use of DNA as a nutrient involves a DNA uptake machinery conserved in naturally transformable species (Figure S1).

Genomic analysis allowed the identification in the genome of *E. coli* of the entire set of genes required for assembly of this machinery (Claverys and Martin, 2003) (Chapter 3, Table 1). Interestingly, these genes have been recently shown to belong to a CRP-S regulon, which is under control of Sxy and CRP (cyclic AMP receptor) proteins, (Sinha *et al.*, 2009) providing support to the view that *E. coli* cells could develop natural competence, although culture conditions that would allow induction of *sxy* have not been identified.

However, among the eight 'competence' genes selected by Palchevskiy and Finkel (2006) to study their possible involvement in the use of DNA as a nutrient, only one, *hofQ*, had a predicted function clearly related to the DNA uptake machinery; HofQ is expected to be crucial for transport of dsDNA across the outer membrane (Figure S1). Moreover, no *sxy* (i.e. major competence regulator) mutant was included in Palchevskiy and Finkel' studies.

Figure S1. Model of DNA uptake in *E. coli* cells. While HofQ (412 aa) is considered the orthologue of *N. gonorrhoeae* PilQ (720 aa) (30% identity over 414 residues), GspD (650 aa) is a PilQ paralogue (25% identity over 278 residues), which was considered as a possible alternative route for dsDNA across the outer membrane, OM; IM, inner (cytoplasmic) membrane.
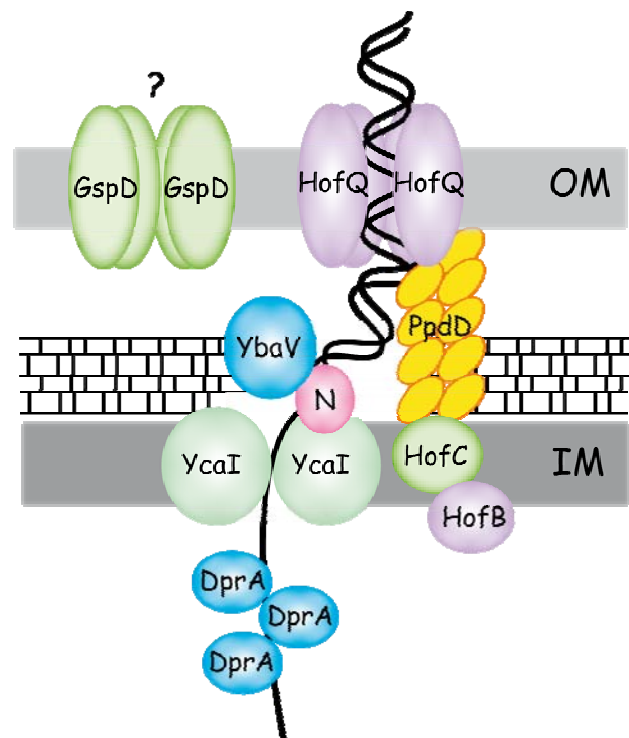
Unfortunately, none of the genes potentially required for dsDNA transport through the periplasm, for conversion of dsDNA into ssDNA, and for internalization of ssDNA into the cytoplasm (Figure S1) were considered in their study (Palchevskiy and Finkel, 2006). This information is important because it would establish whether it is likely that ssDNA fragments are internalized with a 3' to 5' polarity, as demonstrated in the naturally transformable species

Streptococcus pneumoniae (Méjean and Claverys, 1988), (Méjean and Claverys, 1993). Alternatively, dispensability of *ycaI* with respect to the use of DNA as a nutrient by *E. coli* cells would strongly suggest that dsDNA is degraded in the periplasm and that degradation products are then transported into the cytoplasm.

It is also of note that there is a lack of information regarding the behavior of *E. coli* competence regulation mutants. It has only been reported that *sxy* mutant cells display a loss of competitive fitness during long-term stationary-phase coculture with wild type (Sinha *et al.*, 2009), but the ability of *sxy* mutant cells to use DNA as a nutrient has not been investigated. This information would be important because if *sxy* mutants were still able to use DNA, this would strongly suggest that the putative competence regulon of *E. coli*, and therefore the transforming DNA transport machinery, is not involved in DNA uptake.

Role of exonucleases in the use of DNA as a nutrient

Despite the lack of information as to whether ssDNA fragments extracted from exogenous dsDNA are internalized in the cytoplasm of *E. coli* cells (see preceding section), the possible involvement of cytoplasmic single-stranded exonucleases (ExoI, VII, and X and RecJ) in the use of ssDNA as a nutrient was then examined by Palchevskiy and Finkel (2009). Quite surprisingly, inactivation of either *exoI*, *VII* or *recJ* was sufficient to abolish the use of DNA as a nutrient and conferred a 'stationary-phase-specific competition-defective' phenotype to the mutants.

Some of these nucleases display 3'→5' activities while other have the opposite, suggesting that the polarity of degradation was unimportant. On the other hand, as single mutants were unable to use DNA whereas it was predicted that due to functional redundancy, a deficiency phenotype might only appear in multiple mutants, it was proposed that these nucleases function in concert in a heretofore uncharacterized complex (Palchevskiy and Finkel, 2009). Such a complex may account for the apparent unimportance of degradation polarity.

Even more puzzling was the observation that an *exoX* mutant showed an increase in cell density seven time greater than that of the wild type when grown in M63 minimal medium supplemented with 0.1% ultrapure sonicated salmon sperm DNA (Palchevskiy and Finkel, 2009). To account for this observation, it was suggested that ExoX could play a role in a process, such as DNA repair, which directs DNA away from the catabolic system, and that in in the *exoX* mutant, disruption of the complex or pathway relying on ExoX would leave more DNA available for catabolism (Palchevskiy and Finkel, 2009).

Since salmon sperm not *E. coli* DNA was used in these experiments, it is difficult to understand which role this heterologous DNA could play in the repair of *E. coli* chromosomes. Furthermore, the *exoX* mutant despite its increased ability to use DNA as a nutrient displayed the very same 'stationary-phase-specific competition-defective' phenotype as the other exonuclease mutants, i.e. it was outcompeted by the wild type (Palchevskiy and Finkel, 2009). This puzzling observation was not discussed by the authors.

There are thus at least two remarkable exceptions to the proposed connection between the ability to use DNA as a nutrient and competitive fitness. On the one hand, the *yrfD* mutant is unable to use exogenous DNA but is not outcompeted by the wild type (Palchevskiy and Finkel, 2006), and, on the other hand, the *exoX* mutant, which displays an enhanced ability to use DNA compared to wild type, is efficiently outcompeted by the wild type (Palchevskiy and Finkel, 2009).

Suggestions for potentially useful controls

DNase I-digested DNA addition in minimal medium to compensate for the loss of fitness of the mutants. To try to clarify the relationship between the loss of competitive fitness and the proposed inability to use intact DNA as a nutrient, it would be interesting to investigate co-cultures of wild-type and *yhiR* mutant (or other putative DNA uptake mutants) cells in M63 minimal medium containing DNase I-digested DNA as the sole source of carbon. This experiment would constitute an interesting control, as **no loss of competitive fitness of the mutants, including exonuclease mutants, would be expected under such conditions**.

DNase I addition to LB medium to rescue 'stationary-phase-specific competition-defective' mutants. As it was proposed that during long-term incubation in LB medium, DNA is released from dead cells into the medium and serves as a nutrient for the minority of cells that are still alive, it would also be interesting to investigate whether addition of DNase I to LB medium would have any effect on the survival of *yhiR* mutant (or any other putative DNA uptake mutant) cells in coculture with wildtype cells. If the explanation of the loss of competitive fitness by the inability to use intact DNA is correct, then **addition of DNase I should abrogate the loss of competitive fitness of both DNA-uptake and exonuclease mutant cells, and rescue them.**

If addition of Dnase I-digested DNA to minimal medium and of DNase I to LB medium do not rescue the mutants, this would reveal the need to search for alternative explanation(s) for the loss of competitive fitness of these mutants.

DNA degradation in the periplasm followed by import of degradation products? As a control to the study of the ability of their mutants to grow on nucleotides or nucleosides, Palchevskiy and Finkel (2006) showed that *nupC-nupG* mutant cells, which are unable to take up nucleosides, exhibited reduced growth on nucleosides but curiously, neither *nupC* nor *nupG* mutants were tested for their ability to use DNA as a nutrient. **Direct examination of the ability of *nupC-nupG* mutant cells to utilize dsDNA as a sole source of carbon and energy** would provide a clear answer as to **whether ssDNA or nucleosides are transported from the periplasm into the cytoplasm of *E. coli* cells** grown under these conditions.

Conclusions

A careful examination of experiments and data reported in a series of papers dedicated to the use of DNA by *E. coli* cells as a source of carbon and energy (Finkel and Kolter, 2001), (Palchevskiy and Finkel, 2006), (Palchevskiy and Finkel, 2009) raises a number of intriguing questions regarding
- the poor efficiency with which *E. coli* cells use intact DNA compared to DNA degradation products;
- the overall growth yields achieved;
- the variability in growth kinetics;
- the fact that the inability to use intact DNA by stationary-phase-specific competition-defective' mutants is not in itself sufficient to account for their outcompetition by wildtype cells;
- and the observation that the inability to use DNA as a nutrient does not systematically correlates with a loss of competitive fitness (i.e. the *yrfD* mutant which appeared unable to utilize DNA and is not outcompeted by wild-type cells, and the *exoX* mutant, which is efficiently outcompeted by the wild type despite its enhanced ability to use DNA as a nutrient).

It is also intriguing that despite the fact that their working model is based on the uptake of

dsDNA through a machinery conserved in naturally transformable species (Palchevskiy and Finkel, 2006), uptake parameters as documented in naturally transformable species were not considered for the definition of the DNA substrates used in their nutrition experiments. Particularly, the rationale for using sonicated DNA, which is presumably not a good substrate for natural uptake, and enormous concentrations of DNA (in view of the known affinities in natural transformation systems) appeared questionable.

These and other questions (see preceding section) prompted us to plan to study the behaviour of new putative DNA transport mutants, particularly *ycaI* (Chapter 3, Table 1), and to reinvestigate the use of DNA as a nutrient by *E. coli* cells (Chapter 3). The failure to repeat the previously published observations in two (in fact three; Chapter 3, Acknowledgements) independent laboratories did not allow us to confirm that *E. coli* cells are able to use DNA as a source of carbon and energy, and to establish whether ssDNA can be internalized in *E. coli* via the DNA uptake machinery conserved in naturally transformable species.

Concluding remark. In this context, the questions of whether *E. coli* cells use DNA as a source of carbon and energy, and whether they internalize it into their cytosol as ssDNA via a DNA uptake machinery conserved in naturally transformable species will remain pending until an independent laboratory revisits this matter. Meanwhile, one should refer to the so-called nutritional competence of *E. coli* only with caution.

References

Barany, F., and Tomasz, A. (1980) Genetic transformation of *Streptococcus pneumoniae* by heterologous plasmid deoxyribonucleic acid. *J Bacteriol* **144**: 698-709.

Blokesch, M., and Schoolnik, G.K. (2008) The extracellular nuclease Dns and its role in natural transformation of *Vibrio cholerae*. *J Bacteriol* **190**: 7232-7240.

Claverys, J.P., and Håvarstein, L.S. (2007) Cannibalism and fratricide: mechanisms and raisons d'être. *Nat Rev Microbiol* **5**: 219-229.

Claverys, J.P., and Martin, B. (2003) Bacterial "competence" genes: signatures of active transformation, or only remnants? *Trends Microbiol* **11**: 161-165.

Dubnau, D. (1999) DNA uptake in bacteria. *Annu Rev Microbiol* **53**: 217-244.

Dubnau, D. (1991) Genetic competence in *Bacillus subtilis*. *Microbiol Rev* **55**: 395-424.

Finkel, S.E., and Kolter, R. (2001) DNA as a nutrient: novel role for bacterial competence gene homologs. *J Bacteriol* **183**: 6288-6293.

Méjean, V., and Claverys, J.P. (1993) DNA processing during entry in transformation of *Streptococcus pneumoniae*. *J Biol Chem* **268**: 5594-5599.

Méjean, V., and Claverys, J.P. (1988) Polarity of DNA entry in transformation of *Streptococcus pneumoniae*. *Mol Gen Genet* **213**: 444-448.

Palchevskiy, V., and Finkel, S.E. (2006) *Escherichia coli* competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient. *J Bacteriol* **188**: 3902-3910.

Palchevskiy, V., and Finkel, S.E. (2009) A role for single-stranded exonucleases in the use of DNA as a nutrient. *J Bacteriol* **191**: 3712-3716.

Sinha, S., Cameron, A.D., and Redfield, R.J. (2009) Sxy induces a CRP-S regulon in *Escherichia coli*. *J Bacteriol* **191**: 5180-5195.

C . General Discussion

General Discussion

The aim of this thesis was to investigate natural transformation of a proteobacteria γ bacterium *Escherichia coli*, and the possible involvement of competence genes in spontaneous plasmid DNA transformation and in so-called nutritional competence.

Natural plasmid transformation in *Escherichia coli*

The common intestine dweller *E. coli* has traditionally not been regarded as a naturally transformable species (Solomon and Grossman, 1996). However, several reports suggest that *E. coli* can at least take up some DNA under natural conditions. Baur *et al*. documented that transformation of *E. coli* in natural spring water (Baur *et al.*, 1996). Tsen *et al.* and Maeda *et al.* observed transformation of *E. coli* on agar plates with a low concentration of .Ca^{2+} (Maeda *et al.*, 2004; Tsen *et al.*, 2002). As was described in Chapter 1, I established a new transformation protocol which provides further evidence that *E. coli* is capable of spontaneous transformation on the surface of solid Luria-Bertani agar plate (Sun *et al.*, 2006). In this protocol, neither a high concentration of Ca^{2+} nor heat shock or temperature shift is not required to natural transformation of *E. coli* (Sun *et al.*, 2006). My study on natural transformation of *E. coli*, together with previous investigations, establish that plasmid DNA transforms *E. coli* spontaneously under conditions that can be easily found in the environment (Baur *et al.*, 1996; Maeda *et al.*, 2004; Sun *et al.*, 2006; Tsen *et al.*, 2002). Considering that *E. coli* has been widely used in genetic engineering, we should be aware that recombinant plasmids may spread modified genetic material to our environment through natural transformation, which brings potential risks to our environment and health.

Transformation of *E. coli* on solid surfaces

In Chapter 1, I found that *E. coli* can hardly establish competence in liquid cultures and that contact between cells (and possibly with a solid surface) is a pre-requisite for natural transformation. In Chapter 2, I discovered that agar/agarose strongly stimulated plasmid

transformation on plates (Sun *et al.*, 2009). This observation further suggested that contact with a solid surface and/or cell-to-cell contacts stimulate competence in *E. coli*. However, it remains unknown whether/how *E. coli* cells respond to a solid surface to establish competence and how exogenous plasmid DNA interacts with cells on the solid surface.

Effect of cations in LB-agar plates

The addition of divalent cations, such as Ca^{2+}, Mg^{2+} and Mn^{2+}, which induce chemical transformation, does not stimulate plasmid transformation on plates (Sun *et al.*, 2009). This result shows that transformation stimulation by agar or agarose is not due to these divalent cations contained in plates. However, excess of the divalent chelator EGTA suppresses transformation (Sun *et al.*, 2009), indicating that a limited amount of divalent cations is probably required for optimal transformation. The suppressing effect of EGTA could result from   chelation of divalent cations which are essential for the activity of protein(s) involved at some stage(s) in the transformation process, e.g. DNA uptake or plasmid (re)establishment in the cytoplasm.

Effect of pore size on the surface of agar plates

According to the measurement with atomic force microscopy, agarose concentration determines the size of pores on the surface of agarose gel. Pore size in 2% agarose and 5% agarose are 364 nm and 201 nm respectively (Pernodet *et al.*, 1997). Based on data presented in Figure 3 in Pernodet *et al.* (1997), the pore size in 1% agarose should be more than 450 nm. Therefore, plasmid DNA molecule may be more diffusible and thus more difficult to be fixed by *E. coli* cells on the surface of 1% agar/agarose. I attempted to test whether smaller pore size provided by filters limited plasmid DNA movement on plates with 1% agar and thus promote transformation (see Chapter 2 Supplemental Materials 2). However, no transformation improvement was observed. It is possible that the filters introduced additional barriers for DNA uptake, thus preventing a correct evaluation of the pore size hypothesis.

Effect of cell density

In Chapter 2, we found that cell density affects plasmid transformation on plates where biofilm often forms. In *S. pneumoniae*, the competence-stimulating pheromone induces both quorum sensing and competence. It is possible that a high cell density in biofilm produces certain substance(s) to induce competence for plasmid transformation of *E. coli* on plates. A

recent report documented that a putative transformation promoting pheromone may participate in cell-to-cell transformation of *E. coli* (Etchuuya *et al.*, 2011). It would be interesting to identify the pheromone and examine its effect on plasmid transformation with naked DNA.

DNA uptake machinery does not mediate plasmid transformation

In Chapter 2, DNA uptake mechanism was investigated in natural transformation of *E. coli*. Normally, a conserved membrane protein complex for single stranded DNA (ssDNA) passage is responsible for exogenous DNA uptake (Chen and Dubnau, 2004; Johnsborg *et al.*, 2007). To know whether this DNA uptake machine mediates natural plasmid transformation of *E. coli*, I inactivated several genes predicted to encode key components for DNA uptake (Sun *et al.*, 2009). These mutants can not assemble the predicted inner membrane (IM) channel, the outer membrane (OM) channel or the pseudo-pilus for DNA penetration across two membranes (see Fig. 3 in General Introduction). I found that none of these genes were required to plasmid transformation of *E. coli* on plates (Sun *et al.*, 2009), indicating that the classical DNA uptake machinery does not mediate spontaneous plasmid transformation in *E. coli*. As DNA uptake machinery allows only ssDNA to enter into the cytoplasm (Bergé *et al.*, 2003; Méjean and Claverys, 1993), it is unlikely that ssDNA was taken up into *E. coli* cells during natural plasmid transformation.

Two routes for DNA entry into gram negative bacterial cells: one for dsDNA and one for ssDNA?

Since a two-hit kinetics as a function of DNA concentration was detected for monomeric plasmid transformation of *S. pneumoniae* (Saunders and Guild, 1981), it has been proposed that two ssDNA strands are taken up into a cell from two different donor plasmid molecules and can reconstitute a circurlar structure that can be repaired. By contrast, monomeric plasmid transforms *E. coli* with a first order kinetics, indicating that dsDNA from one donor plasmid molecule is sufficient to generate a transformant in *E. coli* (Sun *et al.*, 2009). I therefore

concluded that dsDNA enters into *E. coli* cells. Accordingly, a route for accommodating dsDNA entry should exist. The presence of a complete set of DNA uptake genes (Claverys and Martin, 2003), which could be regulated by the main competence regulator Sxy (Sinha *et al.*, 2009), suggests that there could exist a machinery for ssDNA entry. Therefore, *E. coli* potentially possesses two different routes for dsDNA and ssDNA entry.

In *H. influenzae*, DNA uptake genes are required for natural transformation with chromosomal DNA or a plasmid containing DNA uptake sequence (DUS). However, when this bacterium is transformed with a plasmid containing neither chromosomal fragment or DUS, e.g, RSF0885, the most conserved DNA uptake gene *rec-2*, which encodes a putative IM channel, is not required (Notani *et al.*, 1981). This indicates that some plasmids may not pass through the putative IM channel for ssDNA entry. Therefore, an alternative route for DNA passing across IM could exist in *H. influenzae* (Pifer, 1986).

A different "competence state" for dsDNA uptake?

The competence regulator Sxy controls the expression of components of the DNA uptake machinery in. *H. influenzae* (Cameron and Redfield, 2008; Sinha *et al.*, 2009). Experimental evidence show that Sxy also induces competence in other proteobacteria γ bacteria, e.g. *V. cholerae* and *Actinobacillus pleuropneumoniae* (Bosse *et al.*, 2009; Meibom *et al.*, 2005). In these naturally transformable bacteria, Sxy induces a competence state for assembling DNA uptake machinery to internalize ssDNA. Transcriptome analyses show that competence regulons are activated by over-expressing Sxy in *E. coli* (Sinha *et al.*, 2009). I investigated possible involvement of Sxy in natural plasmid transformation of *E. coli* (Chapter 2 Supplemental Materials). I compared transformation frequency in a *sxy* mutant and its wild-type parent, and found that their transformation frequencies showed no significant difference (Chapter 2 Supplemental Materials). This result reveals that *sxy* is not involved in competence regulation in *E. coli*. Therefore, there could exists a different competence state for dsDNA uptake.

Can *E. coli* use DNA as food?

E. coli has been reported to be able to use DNA as a nutrient and *hofQ*, a DNA uptake gene ortholog predicted to encode an OM porin, is involved in (Finkel and Kolter, 2001; Palchevskiy and Finkel, 2006). I attempted to investigate whether the predicted IM porin YcaI and the competence regulator Sxy were involved in the use of DNA as food using a previously published experimental system (Finkel and Kolter, 2001; Palchevskiy and Finkel, 2006). However, I did not observe that wild type *E. coli* was able to use DNA as a nutrient (Chapter 3). Therefore, the ability of using DNA as a nutrient and the involvement of *E. coli* competence gene orthologues in nutritional competence remain to be established.

Concluding Remarks

To further explore the association between DNA uptake machinery and natural transformation in *E. coli*, it could be interesting to seek first for a confirmation that *E. coli* is able to perform chromosomal transformation by screening a large number of natural isolates with the hope that one of them could develop competence spontaneously. This would have to be done under a large variety of growth conditions (poor versus rich medium, exponential versus stationary phase, etc…) since conditions allowing competence development in a given species are largely unpredictable, as illustrated in the case of *V. cholerae*, which induces competence in response to chitin.

References

Baur, B., Hanselmann, K., Schlimme, W., and Jenni, B. (1996) Genetic transformation in freshwater: *Escherichia coli* is able to develop natural competence. *Appl Environ Microbiol* **62**: 3673-3678.

Bergé, M., Mortier-Barriere, I., Martin, B., and Claverys, J.P. (2003) Transformation of *Streptococcus pneumoniae* relies on DprA- and RecA-dependent protection of incoming DNA single strands. *Mol Microbiol* **50**: 527-536.

Bosse, J.T., Sinha, S., Schippers, T., Kroll, J.S., Redfield, R.J., and Langford, P.R. (2009) Natural competence in strains of *Actinobacillus pleuropneumoniae*. *FEMS Microbiol*

Lett **298**: 124-130.

Cameron, A.D., and Redfield, R.J. (2008) CRP binding and transcription activation at CRP-S sites. *J Mol Biol* **383**: 313-323.

Chen, I., and Dubnau, D. (2004) DNA uptake during bacterial transformation. *Nat Rev Microbiol* **2**: 241-249.

Claverys, J.P., and Martin, B. (2003) Bacterial "competence" genes: signatures of active transformation, or only remnants? *Trends Microbiol* **11**: 161-165.

Etchuuya, R., Ito, M., Kitano, S., Shigi, F., Sobue, R., and Maeda, S. (2011) Cell-to-Cell Transformation in *Escherichia coli*: A Novel Type of Natural Transformation Involving Cell-Derived DNA and a Putative Promoting Pheromone. *PLoS One* **6**: e16355.

Finkel, S.E., and Kolter, R. (2001) DNA as a nutrient: novel role for bacterial competence gene homologs. *J Bacteriol* **183**: 6288-6293.

Johnsborg, O., Eldholm, V., and Havarstein, L.S. (2007) Natural genetic transformation: prevalence, mechanisms and function. *Res Microbiol* **158**: 767-778.

Méjean, V., and Claverys, J.P. (1993) DNA processing during entry in transformation of *Streptococcus pneumoniae*. *J Biol Chem* **268**: 5594-5599.

Maeda, S., Sawamura, A., and Matsuda, A. (2004) Transformation of colonial *Escherichia coli* on solid media. *FEMS Microbiol Lett* **236**: 61-64.

Meibom, K.L., Blokesch, M., Dolganov, N.A., Wu, C.Y., and Schoolnik, G.K. (2005) Chitin induces natural competence in *Vibrio cholerae*. *Science* **310**: 1824-1827.

Notani, N.K., Setlow, J.K., McCarthy, D., and Clayton, N.L. (1981) Transformation of *Haemophilus influenzae* by plasmid RSF0885. *J Bacteriol* **148**: 812-816.

Palchevskiy, V., and Finkel, S.E. (2006) *Escherichia coli* competence gene homologs are essential for competitive fitness and the use of DNA as a nutrient. *J Bacteriol* **188**: 3902-3910.

Pernodet, N., Maaloum, M., and Tinland, B. (1997) Pore size of agarose gels by atomic force microscopy. *Electrophoresis* **18**: 55-58.

Pifer, M.L. (1986) Plasmid establishment in competent *Haemophilus influenzae* occurs by illegitimate transformation. *J Bacteriol* **168**: 683-687.

Saunders, C.W., and Guild, W.R. (1981) Monomer plasmid DNA transforms *Streptococcus pneumoniae*. *Mol Gen Genet* **181**: 57-62.

Sinha, S., Cameron, A.D., and Redfield, R.J. (2009) Sxy induces a CRP-S regulon in *Escherichia coli*. *J Bacteriol* **191**: 5180-5195.

Solomon, J.M., and Grossman, A.D. (1996) Who's competent and when: regulation of natural genetic competence in bacteria. *Trends Genet* **12**: 150-155.

Sun, D., Zhang, Y., Mei, Y., Jiang, H., Xie, Z., Liu, H., Chen, X., and Shen, P. (2006) *Escherichia coli* is naturally transformable in a novel transformation system. *FEMS Microbiol Lett* **265**: 249-255.

Sun, D., Zhang, X., Wang, L., Prudhomme, M., Xie, Z., Martin, B., and Claverys, J.P. (2009) Transforming DNA uptake gene orthologs do not mediate spontaneous plasmid transformation in *Escherichia coli*. *J Bacteriol* **191**: 713-719.

Tsen, S.D., Fang, S.S., Chen, M.J., Chien, J.Y., Lee, C.C., and Tsen, D.H. (2002) Natural plasmid transformation in *Escherichia coli*. *J Biomed Sci* **9**: 246-252.