

## Genetic Transformation in Freshwater: *Escherichia coli* Is Able To Develop Natural Competence

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**Until now, *Escherichia coli* was thought to be unable to develop natural competence, i.e., genetic transformation could be achieved only artificially with the aid of nonphysiological concentrations of calcium ions or by other treatments. We have tested the competence development of *E. coli* through transformation under natural conditions in river water, springwater, and mineral water which contained between 0 and 11 mM Ca<sup>2+</sup>, using pUC18 DNA. The presence of calcium ions at concentrations as low as 1 to 2 mM was sufficient to obtain transformants. Variations in the temperature of incubation were not required for competence development but had an influence on the transformation frequency. Using water from mineral springs originating from calcareous regions, we have obtained transformation frequencies with laboratory strains of *E. coli* similar to those reported for other gram-negative bacteria known to develop natural competence. The competence development of *E. coli* is most probably internally regulated (as for the other gram-negative bacteria), and inadequate conditions chosen for the transformation tests in the laboratory might impair the detection of higher natural transformation frequencies. The results will enhance our knowledge about the fate of laboratory or production strains of *E. coli* cells reaching natural aquatic ecosystems.**

Gene transfer between bacteria in natural ecosystems has gained much attention with the growing concern about the fate of genetically engineered microorganisms released into the environment. Horizontal gene transfer between bacteria in natural aquatic systems has been shown to occur by conjugation (22), transduction (34), transformation (28, 37, 39), and cell contact-mediated transformation, as described by Paul et al. (29). Genetic transformation is a process in which a bacterial recipient can take up exogenous free DNA and incorporate it into its own chromosome by homologous recombination or convert it into an autonomous extrachromosomal replicon. A complete transformation process takes place in four successive steps which are common to all bacteria and necessary for the successful detection of transformants: (i) the development of cell competence, (ii) DNA binding, (iii) DNA uptake and processing, and (iv) phenotypic expression of the new genotype.

Natural bacterial genetic competence is a physiological state which permits the uptake of exogenous high-molecular-weight DNA. Natural competence has been generally differentiated from artificial competence, the latter resulting from physicochemical treatments which force the uptake of the transforming DNA. Strains of many genera of gram-positive and gram-negative bacteria are known to develop natural competence. There have been recent reviews dealing with genetic transformation, competence development, and DNA uptake processes (8, 9, 18, 26, 33, 35, 37, 38).

The development of genetic competence is usually monitored by the detection of transformants. The steps following the development of competence (DNA binding, uptake, processing, and expression) must thus be successful and not limiting if realistic monitoring of competence development is de-

sired. Competence is physiologically regulated and inducible in naturally transformable bacteria. Constitutive competence has been found only in *Neisseria gonorrhoeae* (36). In gram-positive bacteria, competence is usually induced and controlled by competence factors which are secreted into the medium (33). In such cases, the competence is induced as soon as the competence factor has reached a certain concentration (38). In contrast, in gram-negative bacteria, competence is usually internally regulated (26, 38).

The problem with studies of natural competence development is finding the relevant environmental parameters which trigger this induction. Lorenz and Wackernagel (18) have reviewed the known parameters. The bacteria have to be metabolically active, and a shift to unbalanced growth, e.g., by nutrient limitation, can trigger competence development in many gram-negative bacteria. The multiple variations in the required conditions found illustrate the different ecologies of the variety of bacteria chosen. For example, *Azotobacter vinelandii*, a typical soil bacterium, is best transformable after growth in minimal media (24) while *Acinetobacter calcoaceticus*, a ubiquitous human pathogen (opportunistic) usually found on the skin, develops competence in complex as well as minimal media (27).

Metal ions, such as Ca<sup>2+</sup> and Mg<sup>2+</sup>, are key elements for competence induction. Ca<sup>2+</sup> (1 mM) was shown to be necessary for cell growth and competence induction in *Streptococcus pneumoniae* (41). Ca<sup>2+</sup> (0.5 to 1 mM) gave optimal competence development and Mg<sup>2+</sup> was required for the transformation of *A. vinelandii* (25). Moreover, Norris et al. (21) reported that the intracellular calcium concentration in *Escherichia coli* is tightly regulated at 0.1 mM, a level similar to that in eukaryotic cells. A lot of evidence suggests a role for calcium in the regulation of its cell cycle. The artificial transformation of *E. coli* by incubation in highly concentrated solutions of CaCl<sub>2</sub> is well known (4, 19, 20). These facts suggested to us the possibility of natural competence development of *E. coli* during incubation in natural water samples originating from calcare-

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ous regions (rivers, springs, and mineral waters) which contained different  $\text{Ca}^{2+}$  concentrations. In this study, we present evidence that *E. coli* (laboratory strains) becomes competent for transformation with pUC18 plasmid DNA under conditions which prevail in certain natural aquatic ecosystems and that the competence is maintained for several weeks in resting cells.

#### MATERIALS AND METHODS

**Bacterial strains and preparation of the cell suspensions.** *E. coli* K-12 strains HB101 (DSM 1607) and JM109 (DSM 3423) were used and grown in Luria-Bertani medium (32) at 37°C. An overnight culture was used to inoculate fresh cultures which were grown at 37°C to an optical density at 600 nm of 0.6. The cells were centrifuged at  $10,000 \times g$  for 10 min, washed once with water sampled at the natural sites or standardized calcium solution (10 mM  $\text{CaCl}_2$ ), and gently resuspended in the same incubation medium. The centrifugation, washing, and resuspension were performed at the temperature selected for the first incubation of the transformation assay (see below). The pH of the incubation medium after the addition of the cells and at the beginning and until the end of the standardized assay was between 6.5 and 7.5.

**Isolation of plasmid DNA.** Double-stranded supercoiled pUC18 DNA containing an ampicillin resistance gene was isolated from JM109 hosts by utilizing Qiagen columns (Qiagen Inc., Chatsworth, Calif.). DNA concentrations were determined spectrophotometrically at 260 nm.

**Transformation protocols.** Our transformation assay is based on the protocol described by Sambrook et al. (32). The cell suspension was diluted with the precooled incubation medium to yield  $10^8$  cells in 200  $\mu\text{l}$ . Then 25 ng of pUC18 DNA (for a final concentration of 0.125  $\mu\text{g}/\text{ml}$ ) was added, and the sample was further incubated for 20 min at the first incubation temperature. The tubes were then transferred for 10 min to a water bath preadjusted to the second incubation temperature, which was different from the first one if a temperature jump was required. Thereafter, 500  $\mu\text{l}$  of prewarmed Luria-Bertani medium was added and the sample was further incubated for 45 min at the second incubation temperature.

The number of transformants was determined by plating aliquots of the cell suspension on Luria-Bertani plates containing ampicillin (100  $\mu\text{g}/\text{ml}$ ). Plating dilutions on medium without ampicillin gave the total number of cells used for the calculation of the transformation frequency (number of transformants relative to the number of culturable cells). The transformation frequency must be distinguished from the transformation efficiency, which is the number of transformants per 1  $\mu\text{g}$  of transforming DNA. The limit of detection is determined as the reciprocal of the total number of viable (culturable) recipient cells.

In our standard assay, the first temperature was maintained between 0 and 4°C and the second temperature was set at 37°C (standard 0 to 37°C temperature jump). To study the effects of different temperature jumps on the transformation frequency, experiments in which the first or second temperature of incubation was changed were performed.

To study the influence of incubation time on the transformation frequency, experiments were performed with 10 mM  $\text{CaCl}_2$  or springwater with the initial incubation temperature (0 or 10°C) extended up to 21 days. Aliquots were withdrawn periodically, DNA was added, and the transformation protocol was followed as described above (standard 0 to 37°C jump). In another set of experiments, the temperature was maintained at 10°C throughout.

**Water samples used as incubation media for the transformation.** Surface water taken from rivers and springs and bottled mineral waters were used as incubation media for the transformation assays. Incubation in a  $\text{CaCl}_2$  solution (10 mM) made in bidistilled water was done for each experiment as a control. The water samples were sterilized by filtration through nitrocellulose membrane filters (0.22- $\mu\text{m}$  pore size).

The water samples used and their calcium contents are presented in Table 1. Surface water samples were collected in the vicinity of Basel in the calcareous region in the Jura mountains of Switzerland. Water from the rivers Lüssel, Doubs, Lützel, and Birs contains  $\text{Ca}^{2+}$  concentrations of 2 to 2.5 mM. Water samples withdrawn from the rivers Rhine and Wiese, which originate in granitic regions, contain 1.5 and 0.3 mM calcium, respectively. Water from the spring Mariastein, situated in a highly calcareous region near Basel, was tested during a 1-year hydrological cycle. The samples, collected at the spring and 200 m downstream after the  $\text{CaCO}_3$  precipitation front, showed constant  $\text{Ca}^{2+}$  concentrations of 3.3 and 3.1 mM, respectively. The temperature of the water remained between 9.5 and 11.5°C throughout the year.

The use of bottled mineral water was a convenient way to test springwater samples from other regions (France, Switzerland, and Italy). These waters contained 2 to 11 mM  $\text{Ca}^{2+}$ .

**Determination of  $\text{Ca}^{2+}$  concentrations.**  $\text{Ca}^{2+}$  concentrations were determined by atomic emission spectroscopy, and quick estimations with an accuracy of  $\pm 10\%$  were possible with the Merckoquant calcium test (Merck, Darmstadt, Germany).

TABLE 1.  $\text{Ca}^{2+}$  concentrations of water samples and related numbers of transformants<sup>a</sup>

Sample type or source	Sample no.	$\text{Ca}^{2+}$ concn (mM)	No. of transformants
<b>River water</b>			
Wiese (upstream of Basel)	1	0.3	0
Wiese (Basel)	2	0.5	0
Rhine (upstream of Basel)	3	1.4	10
Rhine (downstream of Basel)	4	1.5	5
Doubs (St. Ursanne)	5	2.0	7
Lüssel (Erschwil)	6	1.9	43
Lützel (Röschenz)	7	2.4	137
Birs (Duggingen)	8	2.3	55
Birs (Birsfelden) <sup>b</sup>	9	2.2	20,000
<b>Springwater</b>			
Mariastein <sup>c</sup>	10	3.3	481
Mariastein <sup>d</sup>	11	3.1	280
<b>Bottled mineral water<sup>e</sup></b>			
Evian	12	1.95	12
Henniez	13	2.7	126
Fabia	14	3.1	41
San Pellegrino	15	5.2	472
Passugger	16	6.0	585
Valser	17	10.9	1,510
Aproz	18	11.3	1,822
<b>Control solutions</b>			
$\text{CaCl}_2$			
5 mM	19	5.0	640
10 mM	20	10.0	5,670
50 mM	21	50.0	37,100
$\text{NaCl}$ 50 mM	22	0.0	0
$\text{MgCl}_2$			
25 mM	23	0.0	9
50 mM	24	0.0	121
100 mM	25	0.0	119
Bidistilled water	26	0.0	0
Tap water (Basel)	27	1.5	5
Artificial seawater <sup>f</sup>	28	6.7	10
Luria-Bertani medium	29	0.25	0

<sup>a</sup> The transformation assays were performed with  $10^8$  *E. coli* JM109 cells.

<sup>b</sup> Sampled downstream of the inlet from a wastewater purification plant.

<sup>c</sup> Sampled at the spring.

<sup>d</sup> Sampled after the area of  $\text{CaCO}_3$  precipitation, 200 m downstream from the spring.

<sup>e</sup> Only degassed samples were used.

<sup>f</sup> Sea salt Wimex (Wiegandt GmbH, Krefeld, Germany), 33 g/liter of tap water.

## RESULTS

**Transformation in natural water containing various concentrations of  $\text{Ca}^{2+}$ .** Table 1 gives the number of transformants obtained when *E. coli* JM109 cells were incubated in natural water of known  $\text{Ca}^{2+}$  concentrations. Standard transformation assays were performed with cells harvested at log phase. The transformation frequencies, calculated as the number of transformants per  $10^8$  cells, are shown in Fig. 1, with numbers corresponding to the sample numbers in Table 1. Table 1 reveals that transformants were obtained from all water samples containing a minimum concentration of  $\text{Ca}^{2+}$  of slightly more than 1 mM.  $\text{MgCl}_2$  was able to replace  $\text{Ca}^{2+}$ , but it led to only poor transformation and it had to be provided in high concentrations which do not reflect the composition of natural water. Monovalent  $\text{Na}^+$  ions, bidistilled water, or Luria-Bertani medium alone gave no transformants at all. The highest number of transformants was obtained with water sample 9 (Table 1), which contained only 2.2 mM  $\text{Ca}^{2+}$ , but this partic-

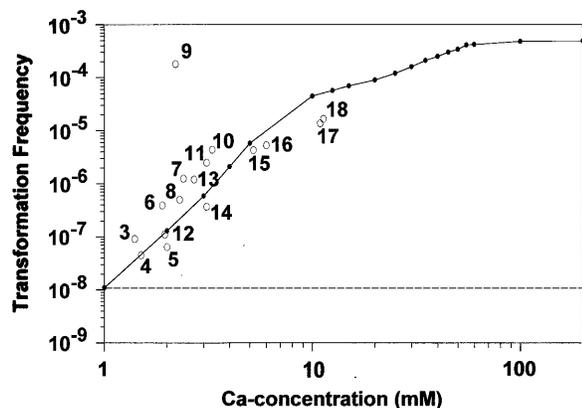


FIG. 1. Transformation frequency as a function of the  $\text{Ca}^{2+}$  concentration. JM109 cells were transformed by pUC18 according to the standard protocol. Values obtained with  $\text{CaCl}_2$  solutions of increasing concentrations (black dots), transformation frequencies calculated from the data presented in Table 1 (open circles), and the limit of detection (dashed line) are shown. The numbers are the sample numbers listed in Table 1.

ular result was not reproducible. The importance of the  $\text{Ca}^{2+}$  for transformation is clearly supported, but there are apparently other, not yet defined compounds present in treated wastewater and in natural water that can inhibit or enhance transformation.

**Correlation between transformation frequency and  $\text{Ca}^{2+}$  concentration.** Standard transformation assays were also performed with solutions of  $\text{CaCl}_2$  of different concentrations, which were used as transformation media. The resulting relationship is shown in Fig. 1. Similar curves have been published previously (19, 20), but calcium concentrations below 10 mM were not assayed. This experiment was also necessary to check the reliability of our transformation protocol and to allow for comparisons with natural water samples. A good correlation between the calcium contents of the water samples and the frequencies of transformation was obtained. These transformation frequencies were also in accordance with those obtained with pure  $\text{CaCl}_2$  solutions.

**Influence of growth phase on transformation.** Transformation was assayed with cells harvested at different growth phases and incubated in  $\text{CaCl}_2$  solutions or in water taken from the

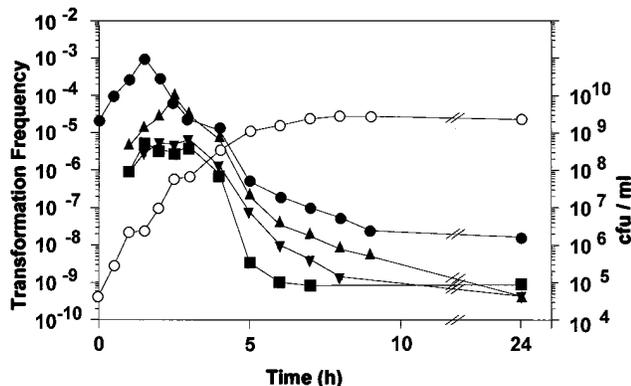


FIG. 2. Effect of growth phase on transformation frequency. Springwater and  $\text{CaCl}_2$  solutions were used for the transformation of JM109 cells taken at various growth stages.  $\circ$ , growth at  $37^\circ\text{C}$ ;  $\blacksquare$ , transformation with Mariastein springwater containing  $3.1 \text{ Ca}^{2+}$ ;  $\blacktriangledown$ ,  $\blacktriangle$ , and  $\bullet$ , transformation with 3, 10, and 50 mM  $\text{CaCl}_2$ , respectively.

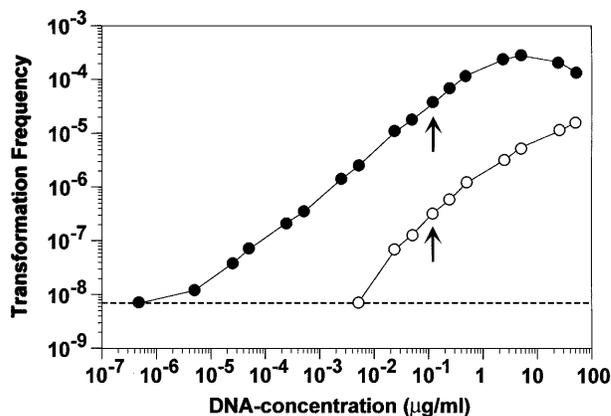


FIG. 3. Effect of DNA concentration on transformation frequency. By the standard protocol, *E. coli* was transformed with pUC18 in 10 mM  $\text{CaCl}_2$  ( $\bullet$ ) or in springwater containing  $3.1 \text{ mM Ca}^{2+}$  ( $\circ$ ). Values are the averages of two experiments. Strains JM109 and HB101 gave similar results. The limit of detection (dashed line) and the DNA concentration used in the standardized assay ( $0.125 \text{ µg/ml}$ ) (arrows) are indicated.

spring Mariastein by the standard assay described above. The results (Fig. 2) show that springwater as well as  $\text{CaCl}_2$  solutions give the highest transformation frequencies with cells harvested during the exponential growth phase. Springwater containing  $3.1 \text{ mM}$  calcium leads to initial transformation frequencies which are very similar to those obtained with a  $3 \text{ mM}$   $\text{CaCl}_2$  solution. Transformation frequencies decreased markedly once the culture shifted from exponential growth to stationary phase, after about 4 h.

**Influence of DNA concentration on transformation frequency.** Transformation assays were performed using different concentrations of transforming pUC18 DNA with cells taken during the exponential phase of growth. Two *E. coli* strains (JM109 and HB101) were tested in water from the spring Mariastein and in  $10 \text{ mM CaCl}_2$ . As expected, the transformation frequencies increased with higher DNA concentrations (Fig. 3). With  $5 \text{ µg}$  of pUC18 DNA per ml, the saturation level with regard to DNA was obtained in  $10 \text{ mM CaCl}_2$  but not in springwater, which contained less  $\text{Ca}^{2+}$ . It is therefore possible to compensate for a low calcium concentration with a high concentration of the transforming DNA. In order to obtain a transformation frequency of  $10^{-6}$ , for example, using the standard assay, a DNA concentration of  $10^{-1} \text{ µg/ml}$  is required in springwater but only  $10^{-3} \text{ µg/ml}$  is necessary in the  $10 \text{ mM CaCl}_2$  solution. Higher transformation frequencies can thus be obtained with all water samples tested by employing higher concentrations of DNA in the assays.

**Influence of temperature shifts on the transformation frequency.** The standard assay includes a temperature shift from  $0$  to  $37^\circ\text{C}$ . This procedure, which is derived from laboratory practice for obtaining high transformation frequencies, does not reflect the conditions in natural aquatic ecosystems. In order to determine to what extent transformation of *E. coli* could occur under natural temperature fluctuations, experiments were performed with variations of the first and/or the second temperature of incubation. The results are given in Fig. 4. As expected from the previous experiments, a  $\text{CaCl}_2$  solution of  $10 \text{ mM}$  always gave higher transformation frequencies (about 100-fold) than Mariastein springwater, which contained only  $3.1 \text{ mM Ca}^{2+}$ . An increase in the first temperature of incubation led to a decrease in the transformation frequencies (Fig. 4). Consequently, the transformation frequencies ob-

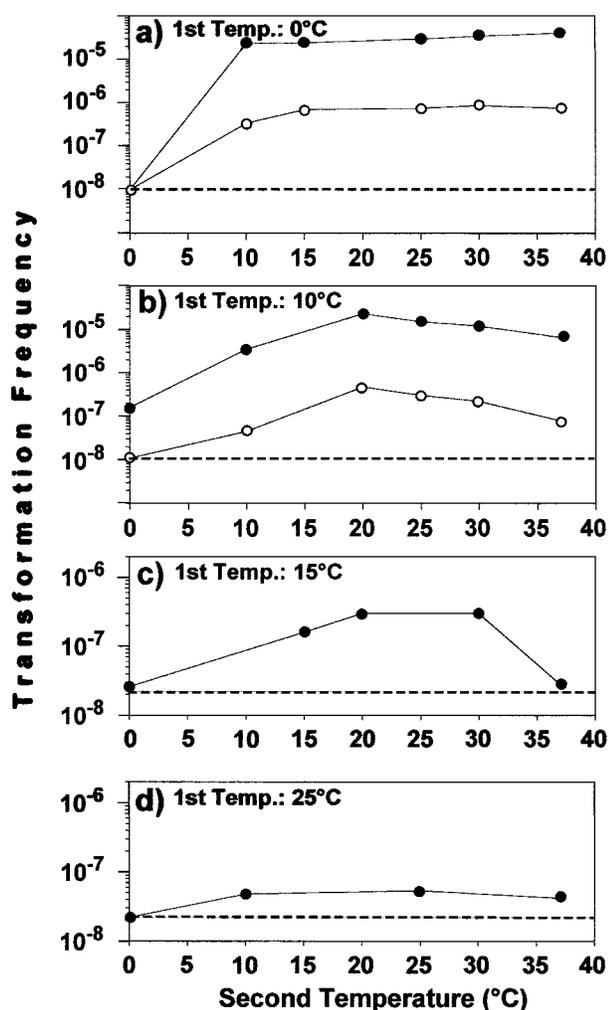


FIG. 4. Influence of temperature variations on transformation frequency. The temperature was shifted from 0, 10, 15, or 25°C (a to d, respectively) to a second temperature indicated on the abscissa. Each point represents the average of two experiments performed with 10 mM  $\text{CaCl}_2$  (●) or springwater containing 3.1 mM  $\text{Ca}^{2+}$  (○). Springwater did not give transformants in the experiments whose results are shown in panels c and d. Strains JM109 and HB101 gave similar results. The limit of detection (dashed line) is indicated.

tained for springwater with 15°C (Fig. 4c) and 25°C (Fig. 4d) as the first incubation temperatures were below the detection limit of our experimental conditions. Figure 4a shows that a temperature shift from 0 to 10°C gave the same transformation frequency as a standard temperature jump from 0 to 37°C. On the other hand, maintaining a constant temperature of 0°C throughout the experiment produced no transformants at all. However, maintaining 10°C for the whole procedure gave transformation frequencies of the same order of magnitude as a temperature shift from 10 to 37°C (Fig. 4b). A temperature shift from 10 to 20°C gave the highest transformation frequencies (Fig. 4b), which were in the same order of magnitude as those obtained with the standard shift from 0 to 37°C (Fig. 4a). A shift from a higher to a lower temperature always diminished transformation frequencies and shifts in the mesothermic temperature range; e.g., shifts from 25 to 37°C or from 15 to 37°C also led to much lower transformation frequencies.

**Maintenance of the competent state during prolonged incubation periods.** Incubation of *E. coli* cells for up to 21 days at

0°C led to an increase of the transformation frequencies (Fig. 5). This was true for 10 mM  $\text{CaCl}_2$  used as an incubation medium (increase from the range of  $10^{-4}$  to  $10^{-1}$ ) and also for Mariastein springwater (increase from  $10^{-6}$  to  $10^{-3}$ ). Similar results were obtained in the presence of the autochthonous microflora which was present in nonfiltered springwater. No significant differences were observed between strains HB101 and JM109. Maintenance of the cells at 10°C in 10 mM  $\text{CaCl}_2$  for 3 weeks led to a decrease in the transformation frequencies from  $10^{-4}$  to  $10^{-5}$  and to a decrease in viability of less than a factor of 10 (not shown). Incubation of the cells in springwater under the same conditions resulted in transformation frequencies above the detection limit for the first 2 days (not shown). In summary, we observed that the competence of *E. coli* was maintained for up to 21 days at low temperatures and in the presence of  $\text{Ca}^{2+}$ . The apparent increase in the transformation frequency is probably due to changing specific DNA concentrations during the incubation period. Since the same amount of DNA (0.125  $\mu\text{g}/\text{ml}$ ) was used in the assays throughout the experiment, the relative amount which became available per viable cell increased with decreasing viability. This variable might have had an influence on the outcome of the experiment since the DNA concentration used in the assays was not at saturation. Balanced conditions with regard to DNA would be difficult to achieve in this case, since a DNA concentration of at least 100  $\mu\text{g}/\text{ml}$  would be required for saturation in a water sample containing only 3.1 mM  $\text{Ca}^{2+}$  (Fig. 3).

## DISCUSSION

The idea that the induction of competence in *E. coli* is physiologically but not physicochemically regulated was put forward by Reusch et al. (30). Those authors observed that the development of competence in *E. coli* is coincident with de novo synthesis and incorporation of poly-beta-hydroxybutyrate (PHB) into the cytoplasmic membranes. In 1995, Huang and Reusch reported that the genetic competence of *E. coli* requires PHB-calcium polyphosphate complexes (14). Calcium plays an important role as a regulatory mediator in prokaryotes

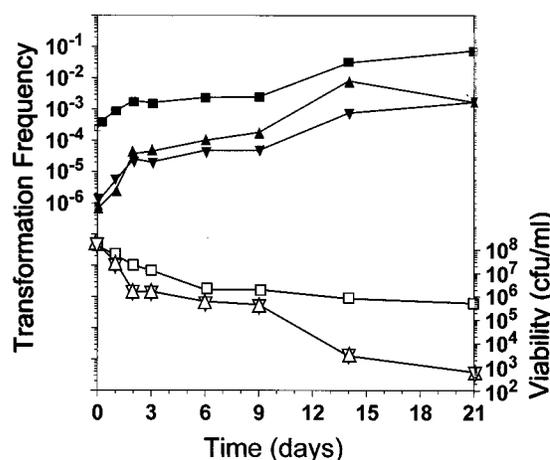


FIG. 5. Maintenance of competence during a prolonged incubation period. The temperature was maintained for 3 weeks at 0°C, and culture aliquots were tested periodically for transformants by the standard transformation procedure. Transformation frequencies in 10 mM  $\text{CaCl}_2$  (■), sterile springwater (▲), or nonsterile springwater (▼) from the spring Mariastein (3.1 mM  $\text{Ca}^{2+}$ ) and viability in 10 mM  $\text{CaCl}_2$  (□), sterile springwater (△), and nonsterile springwater (▽) are shown. Results are averages of two independent experiments with *E. coli* HB101 and JM109.

(21, 23) as well as in eukaryotes (3). Our results show that *E. coli* can develop genetic competence under environmental conditions when in contact with surface water originating from calcareous regions. Calcium concentrations above 1 mM, which are often found in springwater and river water, are sufficient to make *E. coli* cells competent, and no additional competence-promoting factors or buffering substances are needed.

It can be expected that natural environments which are difficult to reproduce in the laboratory might provide optimal conditions for a natural process such as genetic transformation. Use of a cell suspension containing DNA in solution, for example, is a convenient laboratory procedure for transformation assays, but it represents only poorly the environmental situations in which cells often grow in biofilms (5). Even *E. coli* was recently shown to be able to establish itself in biofilms in a chemostat model of a water distribution system (31) or in a biofilm in association with another bacterium in a continuous-flow reactor model system (40). In biofilms, the cell density is normally very high, and many cells may be exposed to free DNA originating from lysed neighboring cells. The lysis of one haploid *E. coli* cell ( $1 \mu\text{m} \times 5 \mu\text{m} \cong 4 \mu\text{m}^3$ ) which contains 4,000 kbp of chromosomal DNA (i.e.,  $2.5 \times 10^9 \text{ Da} \cong 4.2 \times 10^{-9} \mu\text{g}$ ) could produce in its vicinity a DNA concentration of more than 100  $\mu\text{g/ml}$  (a 10-fold dilution factor was assumed for the calculation). The same bacterium containing 100 copies of pUC18 DNA ( $2.9 \times 10^{-10} \mu\text{g}$  of DNA) would provide its neighbor with a pUC18 DNA concentration of 7.4  $\mu\text{g/ml}$ , even after a 10-fold dilution. These concentrations are higher than the ones applied in the usual laboratory assay, and they reach the saturation level for transformation in environments containing 10 mM  $\text{Ca}^{2+}$  (Fig. 3). The DNA saturation level is achieved with 1  $\mu\text{g}$  of DNA per ml in solutions containing 100 mM  $\text{CaCl}_2$  (6). While 3 to 5  $\mu\text{g}$  of DNA per ml would be needed in mineral water containing 10 mM  $\text{Ca}^{2+}$ , more than 100  $\mu\text{g}$  of DNA per ml would be necessary in springwater containing approximately 3 mM  $\text{Ca}^{2+}$  (Fig. 3) in order to saturate  $10^8$  *E. coli* cells. It is conceivable that DNA concentrations of this magnitude can be present in organismically rich biofilms in which transformation between species (especially with plasmid DNA) may contribute to horizontal gene transfer.

Our results confirm those of Bergmans et al. (1), who used 0°C as the initial incubation temperature. Those authors observed also that incubation at this constant temperature gave no transformants at all and that a shift to a higher temperature was necessary. Two years later, the same group (42) reported that successful uptake of pBR322 DNA depended on a temperature shock to 42°C, similar to the one needed for the uptake of chromosomal DNA. Moreover, transformants were obtained after a temperature change from 0°C to a minimum of 15°C for cells grown at 37°C. For cells grown at 22°C, however, competence induction occurred with a smaller temperature shift, from 0 to 10°C. These results illustrate the importance of the procedure applied to grow the cells used in transformation experiments (usually a rather neglected parameter) if environmental conditions are to be simulated. Another confirmation of this statement was given by Inoue et al. (15), who obtained higher transformation frequencies by growing the cells at 18 or 25°C instead of 37°C. Our results extend these findings and show that the highest transformation frequencies which could be obtained under environmental conditions are achieved with small temperature shifts (5 or 10°C) and even in the absence of temperature variations (Fig. 4).

Prolonged survival of *E. coli* in natural aquatic environments is a well-known biological phenomenon (2). In addition, Dagert and Ehrlich (6) have shown that when <1% of *E. coli* cells

were transformed after the incubation time employed in their standard assay, over 20% of viable cells were transformed after 24 h of incubation in  $\text{CaCl}_2$ . Those researchers stimulated competence development during a prolonged incubation by addition of 100 mM  $\text{CaCl}_2$  and saturating concentrations of transforming DNA. Our results confirm that the competence was maintained in springwater during a 3-week incubation in 10 mM  $\text{CaCl}_2$  at 0°C as well as at 10°C and even in the presence of the autochthonous microflora (Fig. 5).

The type of transforming DNA used (chromosomal or plasmid DNA) is a parameter which determines the success of the transformation process per se but not the competence development. We have used a small plasmid to obtain maximal transformation frequencies because it has been demonstrated that the transformation frequencies obtained are inversely proportional to the size of the transforming plasmid (12, 15). On the other hand, the state of the plasmid DNA (supercoiled, circular, linear, or multimeric form) and the presence of cotransformable chromosomal DNA are also key parameters which influence the outcome of the transformation (12, 18, 20, 38). In our case, the method of purification of plasmid DNA led to a majority of supercoiled DNA and open circles free from chromosomal DNA. The absence of multimeric plasmid molecules and of cotransforming chromosomal DNA in the preparation probably led to a minimal estimate of transformation frequencies.

The transformation frequencies obtained under natural conditions which are reported in this work for *E. coli* (i.e.,  $10^{-2}$  to  $10^{-3}$  after a prolonged incubation in Mariastein springwater or  $10^{-5}$  for bottled mineral water) are comparable to the values reported for other bacteria known to be naturally transformable (by plasmid or chromosomal DNA), e.g.,  $10^{-2}$  to  $10^{-3}$  for *Azotobacter vinelandii* OP (7),  $10^{-3}$  to  $10^{-4}$  for *Acinetobacter calcoaceticus* BD413 (16),  $10^{-4}$  for *Bacillus subtilis* 168 (13) and *Vibrio* sp. strain WJT-1C (10), and  $10^{-5}$  for *Pseudomonas stutzeri* JM302 (17) and *Neisseria gonorrhoeae* MS11 (11). Furthermore, it is important to keep in mind that the majority of existing publications are concerned with mutants which are highly prone to transformation (18) or with artificially multimerized plasmids (10). Natural isolates (wild types) belonging to these taxons have been shown to produce a variable proportion of transformable strains (e.g., 89% for *B. subtilis* but <4% for *Acinetobacter calcoaceticus*), and those strains usually show very low transformation frequencies (18).

We have used laboratory strains of *E. coli* and pUC18 DNA since we are interested in assessing the fate of genetically engineered bacteria when they are released into aquatic ecosystems. On the basis of these results, it seems legitimate to presume that the natural development of genetic competence in wild-type *E. coli* is biologically possible. In this case, the transformation process will be highly dependent on the phenotypical characteristics of each strain and on the type and characteristics of the transforming DNA.

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