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Natural Genetic Transformation of Clinical Isolates of *Escherichia coli* in Urine and Water

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Transfer of plasmid-borne antibiotic resistance genes in *Escherichia coli* wild-type strains is possible by transformation under naturally occurring conditions in oligotrophic, aquatic environments containing physiologic concentrations of calcium. In contrast, transformation is suppressed in nitrogen-rich body fluids like urine, a common habitat of uropathogenic strains. Current knowledge indicates that transformation of these *E. coli* wild-type strains is of no relevance for the acquisition of resistance in this clinically important environment.

Horizontal gene transfer is a recognized process that allows the rapid spread of antibiotic resistance genes inside and outside of hospitals, thereby impeding antimicrobial chemotherapy (17). Resistance determinants are readily acquired and disseminated within and among bacterial populations by (i) conjugation, (ii) transduction, and (iii) transformation, virtually without barriers between species (6, 12). Natural genetic transformation is characterized by the uptake of free DNA by a recipient bacterium, its chromosomal integration or extrachromosomal stabilization, and its expression, which leads to a new phenotype (7, 11). Calcium appears to play a pivotal role for the development of bacterial competence, an inducible property of many bacterial species and a prerequisite for transformation, in both gram-positive and gram-negative bacteria (18, 19, 25). Laboratory strains of *Escherichia coli* were shown to be transformable by a nonphysiological Ca^{2+} concentration of 100 mM (9, 22). Recombination- and DNase-deficient laboratory strains are generally used, with a temperature shift from 0 to 37°C after the addition of free DNA (9, 22, 27). Such conditions are never encountered in the original biotope of *E. coli* (i.e., the mammalian gastrointestinal and urogenital tracts). Consequently, this species was not considered to be transformable in its natural habitats (11, 26). However, there have been indications that the induction of competence in *E. coli* is controlled physiologically rather than physicochemically (4), and natural transformation of laboratory strains of *E. coli* has been demonstrated to occur in river, spring, and mineral water (2) and in foodstuffs (1) but not yet in the body fluids of mammals. Our intention was to evaluate whether wild-type clinical isolates of *E. coli* were naturally transformable in freshwater, as has already been reported for laboratory strains, and to evaluate the impact of transformation on the dissemination of antibiotic resistance genes under conditions of clinical significance.

Bacterial strains and plasmid DNA. Eight ampicillin-sensitive, nonpathogenic *E. coli* isolates from sputum or swab samples of the oropharynxes of hospitalized patients with sinusitis or otitis were chosen at random without additional criteria of selection. In addition, we tested four pathogenic strains which were isolated from two patients with urogenital infections. From each patient, a single strain was isolated from feces (S 988 and S 1018) and from urine (U 988 and U 1018), all of which showed identical restriction fragment length polymorphism banding patterns. *E. coli* strain DH5α (number C2007-1; Clontech, Palo Alto, Calif.) was used as a control in each experiment. Supercoiled pUC18 (Roche, Vienna, Austria) and pGFP (Clontech number 6097-1) plasmid DNAs were purified by anion-exchange chromatography (Qiagen, Hilden, Germany).

Transformation assays. Transformation assays were performed as described by Sambrook et al. (22). Briefly, log-phase cells were harvested and the number of viable cells per milliliter (usually 10^8 cells/ml) was determined by agar plating of serial dilutions. The bacteria were washed with the selected water samples or $CaCl_2$ solutions (0 to 100 mM), resuspended in 200 μl of the same solution, and incubated for 1 h (test range, 1 min to 72 h) at the appropriate temperature. Then, supercoiled pUC18 or pGFP plasmid DNA was added at various concentrations ranging from 1.25 ng/ml to 1.25 μg/ml and for different time periods (1 to 60 min). Most of the experiments were performed with DNA incubation for 10 min. A standard shift from 0 to 37°C was applied, usually for 10 min. After addition of 500 μl of Luria-Bertani broth, all samples were incubated at 37°C for 45 min, and 100 μl of the transformation mix was transferred onto Luria-Bertani agar plates containing ampicillin (100 μg/ml). The transformation frequency (T_f) was established by calculating the ratio of the number of transformants per viable cell per milliliter (detection limit, 10^{-7}). The transformation efficiency (T_e) was calculated as the number of transformants per microgram of plasmid DNA applied in a volume of 1 ml. The mean number of

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transformants obtained during three transformation assays was used for calculation.

Transformation assays in human urine were performed as described above, with the exception that all incubations were performed at 37°C in human urine in order to mimic natural conditions. Void urine was collected from healthy volunteers and sterilized by filtration (Millex-GV; Millipore, Bedford, Mass.). For some experiments, urine was treated at 80°C for 10 min to inactivate the DNases. Ca^{2+} concentrations were measured with a calcium detection kit, 587-A, from Sigma (St. Louis, Mo.); pH was determined with pH indicator strips (Merck, Darmstadt, Germany). The bacteria were incubated in 1 ml of urine on a thermomixer for 24 h. They were then pelleted, washed with fresh pretreated urine, resuspended in 500 μl of urine, and incubated for another 4 h under vigorous shaking. After addition of plasmid DNA to 200 μl of the preincubation solution (final concentration, 125 ng/ml), the bacteria were incubated for 24 h. A 500-μl volume of fresh urine was then added, and bacterial growth was enabled for an additional 4 h. Transformants were detected as described above.

Transformation in $CaCl_2$ solutions and in mineral water. As is shown in Table 1, three (EC1, EC2, EC7) out of the eight nonpathogenic *E. coli* isolates persistently yielded transformants upon application of the standard procedure. The other strains either were not transformable (EC3, EC6) or showed fluctuating transformation (EC4, EC5). All four pathogenic strains yielded transformants in three consecutive experiments. EC1 always showed the highest number of transformants. With this strain, competence could be induced with a $CaCl_2$ concentration as low as 1 mM, but T_fs were always modest percentages of the values obtained with the laboratory strain DH5α (11% for 100 mM $CaCl_2$, 3% for 50 mM, 1% for 10 mM). Transformants could be induced with EC1 and DH5α by incubation in bottled, carbonized mineral water (Preblauer, Austria), which contained 2.9 mM Ca^{2+}. No transformants were observed when the strains were incubated in other mineral waters with lower Ca^{2+} contents, in Coca Cola, or in orange or multivitamin juice.

Additional parameters influencing transformability. As is shown in Table 2, 1 min of incubation in $CaCl_2$ was sufficient to generate transformants, indicating that induction of competence is a rapid process. A steep rise in T_e was detectable after 5 min of incubation with EC1 and after 10 min with DH5α. With EC1, 1.25 ng of DNA/ml was sufficient to generate transformants when it was incubated in $CaCl_2$ at a concentration of 10 mM or higher. With 100 mM $CaCl_2$ and 10^9 cells/ml, the T_f increased with rising DNA concentrations whereas the efficiency of DNA transfer remained constant, as one expects in a system not saturated with DNA. With DH5α (10^8 cells/ml), the T_e decreased with rising DNA concentrations, but the T_f essentially remained stable. If one assumes that wild-type and laboratory strains behave similarly in this scenario, what we observed may be explained by a surplus of plasmid molecules compared to the number of competent cells available in a DNA-saturated environment.

Transformants could also be detected after temperature shifts from 25 to 37°C (but not with constant incubation at 25 or 37°C) with a minimum of 10 mM $CaCl_2$. Unlike with DH5α, the number of EC1 transformants did not increase along with

TABLE 1. Transformation of wild-type *E. coli* strains and DH5α[a]

Strain	No. of transformants/ml (mean ± SD)[b]	Transformation result in:											
		Water with $CaCl_2$ at the following concn (mM):								Mineral water[c]		Urine[d]	
		1		10		50		100					
		T_f[e]	T_e	T_f	T_e	T_f	T_e	T_f	T_e	T_f	T_e	T_f
Nonpathogenic strains													
EC1	$2,296 \pm 240.89$	3.0×10^{-8}	2.7×10^{1}	4.0×10^{-7}	3.2×10^{2}	6.8×10^{-6}	5.4×10^{3}	2.3×10^{-5}	1.8×10^{4}	7.0×10^{-8}	5.6×10^{1}	0[f]	
EC2	238 ± 97.83	0	0	1.3×10^{-7}	1.1×10^{2}	1.2×10^{-6}	9.3×10^{2}	2.4×10^{-6}	1.9×10^{3}	ND[g]	ND	ND	
EC7	10 ± 7.07	0	0	0	0	7.0×10^{-8}	5.3×10^{1}	1.0×10^{-7}	8.0×10^{1}	ND	ND	ND	
Pathogenic strains													
S 988	12 ± 10.95	ND	ND	ND	ND	ND	ND	1.2×10^{-7}	9.6×10^{1}	ND	ND	0	
U 988	24 ± 5.48	ND	ND	ND	ND	ND	ND	2.4×10^{-7}	1.9×10^{2}	ND	ND	0	
S 1018 A_{II}	24 ± 15.17	ND	ND	ND	ND	ND	ND	2.4×10^{-7}	1.9×10^{2}	ND	ND	0	
U 1018 A_{II}	44 ± 32.09	ND	ND	ND	ND	ND	ND	4.4×10^{-7}	3.5×10^{2}	ND	ND	0	
Laboratory strain DH5α	$20,692 \pm 651.59$	0	4.1×10^{-5}	3.3×10^{4}	2.0×10^{-4}	1.6×10^{5}	2.1×10^{-4}	1.6×10^{5}	7.0×10^{-8}	5.6×10^{1}	0		

[a] A standard transformation protocol was applied (22). After the addition of pUC18 DNA, the temperature was shifted to 37°C for 10 min.
[b] The number of colonies per milliliter for the transformation assay was obtained during an initial screening for transformable clinical isolates of *E. coli*. Values are the means of results from five experiments.
[c] Mineral water contained 2.9 mM $CaCl_2$.
[d] Urine contained 0.3 to 4.5 mM $CaCl_2$. Strains were incubated at 37°C throughout the assay without the addition of $CaCl_2$.
[e] T_f, number of transformants per viable cell ($\sim 10^8$ cells/ml).
[f] Zero indicates that results were below the limit of detection.
[g] ND, not determined.

TABLE 2. Parameters influencing transformation of *E. coli* wild-type and laboratory strains in water[a]

Parameter	EC1 (wild type)				DH5α (laboratory strain)			
	10 mM $CaCl_2$		100 mM $CaCl_2$		10 mM $CaCl_2$		100 mM $CaCl_2$	
	T_f[b]	T_e	T_f	T_e	T_f	T_e	T_f	T_e
Duration of incubation in $CaCl_2$ (min)								
1	3.0×10^{-8}	2.4×10^{1}	0[c]	0	4.3×10^{-6}	3.4×10^{3}	1.2×10^{-4}	9.8×10^{4}
5	2.0×10^{-7}	1.6×10^{2}	0	0	4.5×10^{-6}	3.6×10^{3}	1.4×10^{-4}	1.1×10^{5}
10	2.3×10^{-7}	1.8×10^{2}	1.7×10^{-7}	1.4×10^{2}	3.3×10^{-5}	2.7×10^{4}	2.9×10^{-4}	2.3×10^{5}
30	5.3×10^{-7}	4.2×10^{2}	1.2×10^{-6}	9.4×10^{2}	4.1×10^{-5}	3.3×10^{4}	3.2×10^{-4}	2.6×10^{5}
DNA concentration (ng/ml)[d]								
1.25	8.0×10^{-8}	6.4×10^{4}	5.0×10^{-8}	4.0×10^{4}	1.1×10^{-6}	8.6×10^{4}	1.0×10^{-4}	8.2×10^{6}
12.5	3.8×10^{-7}	3.0×10^{4}	3.1×10^{-7}	2.5×10^{4}	1.1×10^{-5}	9.1×10^{4}	1.4×10^{-4}	1.1×10^{6}
125[e]	1.9×10^{-6}	1.9×10^{4}	2.6×10^{-6}	2.1×10^{4}	2.2×10^{-5}	2.2×10^{4}	2.0×10^{-4}	1.7×10^{5}
Temp shift from 25 to 37°C	1.0×10^{-7}	8.0×10^{1}	1.0×10^{-7}	8.0×10^{1}	3.0×10^{-8}	2.4×10^{1}	2.2×10^{-5}	1.8×10^{4}

[a] A standard transformation protocol (22) was applied (100 mM $CaCl_2$, 125 ng of pUC18 DNA/ml, temperature shift from 0 to 37°C) unless otherwise stated. Results are means of values from triplicate experiments.
[b] T_f, number of transformants per viable cell (~10^8 cells/ml).
[c] Zero indicates that results were below the limit of detection.
[d] For EC1, 10^9 cells/ml were used for this parameter.
[e] With 10 mM $CaCl_2$, 100 ng of DNA/ml was used.

the concentration of calcium, indicating that the transformability of wild-type strains is more stringently controlled than that of the laboratory strain by the kind of temperature shift.

Transformation in human urine. No transformant was detected with any of the uropathogenic strains, nonpathogenic strains, or strain DH5α in urine (pH 6.8 to 7.5, heat inactivated and nontreated) with constant incubation at 37°C (Table 1). Agarose gel analysis of the pGFP plasmid indicated complete DNA degradation within 5 to 10 min in nontreated urine. Variations of the transformation protocol (i.e., incubation of *E. coli* strains on ice for 24 h prior to addition of plasmid DNA, heat shock at 0 to 37°C, and use of plasmid DNA concentrations of up to 1.25 μg/ml) also did not lead to the formation of transformants. A single positive transformation in one out of three experiments could be detected with DH5α (T_f, 3.0×10^{-8}; T_e, 2.7×10^{1}) after DNase inactivation (by heat) and after the urine sample was spiked with calcium (final Ca^{2+} concentration, 12 mM).

Relevance of different horizontal gene transfer mechanisms for the dissemination of antibiotic resistance. The transfer of antibiotic resistance genes in *E. coli* by conjugation in soil (3), protozoa (23), body fluids (13), and the intestine (16) has been reported and most probably plays a decisive role in the spreading of resistance genes in clinical settings (24). Concerning the phenomenon of transformation in body fluids, the data are scarce. Natural transformation of gram-positive pathogens like *Streptococcus pneumoniae* has been unequivocally demonstrated for humans (21) and mice (5, 20). The development of genetic competence and natural transformation have been shown for *Bacillus subtilis* in milk (29) and for *Streptococcus gordonii* in human saliva (15). Inhibitory effects of rumen fluid and ovine saliva on the development of the competence of *Streptococcus bovis* have also been described (14). Bauer et al. (1) have demonstrated *E. coli* T_fs below 10^{-6} in milk. Recently, vesicle-mediated transfer of virulence genes has been reported, a process which may facilitate the dissemination of DNA fragments in DNase-rich environments like urine (28).

We have detected the transformation of *E. coli* wild-type strains in water at Ca^{2+} concentrations as low as 1 to 2.9 mM, which are readily available in mineral waters and body fluids (proximal ileum, 4.2 mM; urine, 5.9 mM; saliva, 2.3 mM) (10). However, transformants were not detectable in urine. DNA degradation severely impairs the uptake of free DNA in DNase-rich body fluids like urine, but an additional process functioning against transformation can be inferred. (i) A strong correlation has been found between the T_e and the formation of poly-β-hydroxybutyrate (PHB)–calcium–polyphosphate complexes, which are presumably channels for DNA transfer in the cytoplasmic membranes of *E. coli* cells (8). (ii) PHB synthesis is also a way to store carbon in an osmotically inert form when nitrogen becomes the limiting growth factor. (iii) Induction of competence preferentially occurs during starvation caused by a nutrient imbalance, i.e., when the C/N ratio is high (2, 11). Thus, the absence of transformation in urine may be due to the fact that *E. coli* grows initially in a nutrient-rich medium (>10^9 CFU/ml) without the need for PHB synthesis. Under starvation conditions during stationary phase, a low C/N ratio in urine is expected. Neither condition is favorable for the induction of competence. The situation may be reversed in milk that contains an abundance of sugar, leading to a C/N ratio greater than 1 at starvation.

Conclusion. The transformability of *E. coli* (clinical isolates and laboratory strains) is restricted by the environmental conditions prevailing in its habitat. Although genetic transformation of *E. coli* may occur under natural conditions in aquatic, calcareous environments, urine suppresses the rate of transformation to below the detection limit. This observation indicates that natural transformation of *E. coli* wild types is most probably of no relevance for the acquisition of antibiotic resistance determinants in body fluids like urine. Resistant phenotypes may be spread predominantly by bacterial conjugation and vesicle-mediated transfer in clinical settings. However, we cannot rule out the occurrence of transformation in other

natural environments (e.g., mammalian intestine) or for other pathogroups of *E. coli*.

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