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Thematic Issue on Horizontal Gene Transfer

Lack of detectable DNA uptake by bacterial gut isolates grown *in vitro* and by *Acinetobacter baylyi* colonizing rodents *in vivo*

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Biological risk assessment of food containing recombinant DNA has exposed knowledge gaps related to the general fate of DNA in the gastrointestinal tract (GIT). Here, a series of experiments is presented that were designed to determine if genetic transformation of the naturally competent bacterium *Acinetobacter baylyi* BD413 occurs in the GIT of mice and rats, with feed-introduced bacterial DNA containing a kanamycin resistance gene (*npt*II). Strain BD413 was found in various gut locations in germ-free mice at 10^3–10^5 CFU per gram GIT content 24–48 h after administration. However, subsequent DNA exposure of the colonized mice did not result in detectable bacterial transformants, with a detection limit of 1 transformant per 10^3–10^5 bacteria. Further attempts to increase the likelihood of detection by introducing weak positive selection with kanamycin of putative transformants arising *in vivo* during a 4-week-long feeding experiment (where the mice received DNA and the recipient cells regularly) did not yield transformants either. Moreover, the *in vitro* exposure of actively growing *A. baylyi* cells to gut contents from the stomach, small intestine, cecum or colon contents of rats (with a normal microbiota) fed either purified DNA (50 μg) or bacterial cell lysates did not produce bacterial transformants. The presence of gut content of germfree mice was also highly inhibitory to transformation of *A. baylyi,* indicating that microbially-produced nucleases are not responsible for the sharp 500- to 1 000 000-fold reduction of transformation frequencies seen. Finally, a range of isolates from the genera *Enterococcus, Streptococcus* and *Bifidobacterium* spp. was examined for competence expression *in vitro*, without yielding any transformants. In conclusion, model choice and methodological constraints severely limit the sample size and, hence, transfer frequencies that can be measured experimentally in the GIT. Our observations suggest the contents of the GIT shield or adsorb DNA, preventing detectable exposure of feed-derived DNA fragments to competent bacteria.

Keywords: horizontal gene transfer / natural transformation / gastrointestinal tract / gnotobiotic / DNA persistence

INTRODUCTION

DNA macromolecules are continually introduced into the gastrointestinal tract (GIT) as a natural part of food. It has been estimated that humans digest 0.1 to 1 mg DNA per day (Doerfler, 2000). Whereas the majority of feed-derived DNA is broken down during digestion (Martín-Orúe et al., 2002; Palka-Santini et al., 2003; Tony et al., 2003), several studies have now shown that minor proportions of feed-derived DNA survive immediate degradation and reach the bloodstream in various animals (Deaville and Maddison, 2005; Einspanier et al., 2001; Jennings et al., 2002; Schubbert et al., 1994; 1997; 1998) or are detectable as minor fragments in faeces (Chowdhury et al., 2003a; 2003b; 2004; Wilcks et al., 2004). The fate of chromosomal DNA in the gastrointestinal tract (GIT) of humans and animals has recently received increased attention due to the introduction of novel ingredients derived from genetically modified organisms (GMOs) in the food chain (Sharma et al., 2004; 2006). Biological risk assessment of GMOs has exposed knowledge gaps related to how DNA is degraded, or

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survive degradation in various compartments of the GIT. These gaps include a lack of a precise understanding of (i) the amount, size and stability of DNA present in various types of raw and processed food; (ii) how the intracellular location and protein interactions affect DNA stability and degradation; (iii) the relative contribution of and specific activities of the degradation mechanisms and processes present in the GIT; and (iv) a comprehensive understanding of the fragment size distribution of DNA present in various food sources and digestive compartments in mammals. It should be emphasized that these knowledge gaps are not specifically linked to the consumption of recombinant DNA, but encompass the fate of all DNA molecules that enter the GIT.

Previous studies (Forsman et al., 2003; Hohlweg and Doerfler, 2003; Klotz et al., 2002) indicate that food-derived DNA fragments are routinely exposed to epithelial cells of the GIT. It can be reasonably assumed that the GIT microbiota also will be exposed to and utilize feed-derived DNA as a nutrient source. Such DNA exposure can, if competent bacteria are present in the GIT, also lead to genetically transformed bacteria. The colon, with its high nutrient levels, bacterial population density and growth rate, is hypothesized to be an important environment for gene transfer (Mercer et al., 1999a; 1999b; 2001). Yet, few studies have unambiguously shown HGT to take place in the GIT. Whereas some evidence of conjugal gene transfer in the GIT has been presented, we are not aware of any study that has demonstrated natural transformation in the lower GIT (Netherwood et al., 1999). Most published studies have focused on oral bacteria (Duggan et al., 2000; 2003). For instance, it has been reported that the naturally competent *Streptococcus gordonii* DL1 could be transformed by plasmid DNA in saliva (Mercer et al., 1999b; 2001). Other studies examining *in vivo* transfer in gut samples from different mice and rat models have not been able to detect bacterial transformants (Kharazmi et al., 2003; Wilcks et al., 2004). One DNA consumption study in humans (including ileostomists; individuals in which the terminal ileum is resected and the digesta is diverted *via* a stoma to a colostomy bag) indicated that horizontal transfer of recombinant DNA from GM soya to the microbiota of the small bowel had taken place in the past. The DNA sequence data obtained did not, however, allow a mechanistic understanding of the putative HGT event (Netherwood et al., 2004).

Several bacterial species that normally reside in the GIT have been shown to develop competence for natural transformation *in vitro* (Lorenz and Wackernagel, 1994; Mercer et al., 1999a), and in various models of the upper GIT (Duggan et al., 2000; Mercer et al., 1999; 2001) suggesting that fragments of food-ingested DNA can be taken up by bacteria in the GIT by natural transformation. The frequent use of antibiotic resistance genes as markers in genetically modified plants has put forward the question whether such resistance genes can transform pathogenic bacteria present in the GIT (Chambers et al., 2002; Nielsen et al., 2005). Whether HGT from GM food sources into exposed GIT bacteria occurs at all, or will create biosafety relevant adverse impact, is thus debated (Bensasson et al., 2004; EFSA, 2004; Nielsen and Townsend, 2004). It remains unclear to what extent bacteria express competence for natural transformation in the GIT systems of higher organisms (Nielsen and Townsend, 2004; Thomas and Nielsen, 2005).

In this study, the potential for a range of well-characterized anaerobic and aerobic human gut isolates to develop competence *in vitro* is examined. Moreover, a series of experiments is presented that determines to what extent *Acinetobacter baylyi*, a frequently used model bacterium for natural transformation, is capable of DNA uptake during colonization of the GIT of gnotobiotic rodents.

RESULTS

Colonization by and transformability of *A. baylyi* in the GIT of rodents

As the *A. baylyi* strain BD413 is known to develop competence during growth, it was examined whether conditions were present in the GIT that lead to both bacterial growth and establishment of *A. baylyi* cells at high numbers. The *A. baylyi* cells colonized the different parts of the GIT of mice and rats to various extent; as summarized in Table 1. Differences in initial inoculum levels, choice of animal model and sample storage were important for the colonization patterns observed.

In experiment series 1 (see Materials and Methods for details on the different experiment series), *A. baylyi* cells were shown to colonize the colon of two germfree mice at high levels, typically 10^8 CFU per gram sample material. The samples were taken 46 h after administration of a bacterial solution by gavage feeding (Tab. 1). Because the colonization level obtained was sufficient to detect low transformation frequencies (*i.e.* around 1 transformant per 10^8 bacterial cells sampled), experiment series 2 was initiated.

In the second series of experiments, four rats given a bacterial solution by gavage feeding were, after 24 h, given by gavage feeding a single dose of 50 μg DNA (obtained from of *A. baylyi* strain KTG conferring *nptII*-mediated kanamycin-resistance). Two of the rats also received a low dose of the antibiotic kanamycin in the drinking water during the experiment. The latter antibiotic treatment was done to enrich for rare bacterial transformants potentially arising in the rat GIT. *In vivo* uptake

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Table 1. Colonization level of *Acinetobacter baylyi* in different parts of the gastrointestinal tract (GIT) of germfree mice and rats (CFU per g wet weight GIT sample).

Experiment/animal #	Stomach	Cecum	Small intestine	Large intestine
Experiment series 1				
Mouse 1, 2 mean	$2.9 (\pm 2.4) \times 10^5$	$6.5 (\pm 5.9) \times 10^8$	$2.6 (\pm 1.2) \times 10^8$	$6.1 (\pm 4.1) \times 10^8$
Experiment series 2				
Rat 1, 2 mean	1.3×10^2	1.6×10^4	1.3×10^2	6.9×10^3
Rat 3, 4 mean[a]	0	0	0	0
Experiment series 3				
Mouse 2	46 (± 138)	$2.0 (\pm 4.0) \times 10^3$	$2.4 (\pm 1.8) \times 10^3$	$2.6 (\pm 2.1) \times 10^4$
Mouse 4	0	$9.7 (\pm 1.3) \times 10^3$	$3.2 (\pm 2.4) \times 10^5$	$1.1 (\pm 1.0) \times 10^5$
Mouse 6	0	$1.7 (\pm 0.6) \times 10^3$	$3.5 (\pm 1.7) \times 10^5$	$7.4 (\pm 4.2) \times 10^4$
Mouse 5	0	0	0	0
Mouse 1, 3	0	0	0	0
Experiment series 4[b]				
Mouse 1				
MRS medium	$1.0 (\pm 0.6) \times 10^6$	$8.3 (\pm 3.0) \times 10^5$	0	$1.7 (\pm 1.0) \times 10^6$
LA_{Rif-} medium	0	0	0	0
Mouse 2				
LA	$2.1 (\pm 3.3) \times 10^5$	$1.4 (\pm 0.6) \times 10^5$	0	$1.2 (\pm 0.5) \times 10^5$
LA_{Rif}	$2.4 (\pm 3.8) \times 10^4$	$2.0 (\pm 1.2) \times 10^4$	0	$2.3 (\pm 1.2) \times 10^4$
Mouse 3				
LA	$4.6 (\pm 1.2) \times 10^3$	$1.8 (\pm 0.8) \times 10^6$	0	$1.2 (\pm 1.9) \times 10^5$
LA_{Rif}	$1.5 (\pm 0.4) \times 10^3$	$2.7 (\pm 1.5) \times 10^4$	0	$4.0 (\pm 1.7) \times 10^3$
Mouse 4				
LA_{Rif}	$4.1 (\pm 0.7) \times 10^3$	$9.2 (\pm 1.1) \times 10^3$	0	$8.2 (\pm 2.8) \times 10^5$
Mouse 5				
LA	0	0	0	0
Experiment series 5				
Mouse 1	0	$7.0 (\pm 3.0) \times 10^5$	0	$1.0 (\pm 0.4) \times 10^6$
Mouse 2	0	$2.0 (\pm 0.9) \times 10^6$	0	$1.0 (\pm 0.5) \times 10^6$

[a] With kanamycin (10 $mg.L^{-1}$) added to drinking water subsequent (24 h) to DNA exposure.
[b] Co-cultivation experiment where the mice received both strains BD413 and *Lactobacillus rhamnosus* strain LGG (mouse 1), strains BD413 and *Escherichia coli* DH5-alfa (mouse 2), strains BD413 and *Pseudomonas stutzeri* JM303 (mouse 3), strain BD413 only (mouse 4) and no bacterial inoculum (mouse 5).

of the *A. baylyi* KTG DNA was not observed among the sampled bacteria in this experiment. Surprisingly, the bacterial inoculum did not result in colonization of the rat GIT at the same high level as seen in gnotobiotic mice. Due to the low bacterial colonization level obtained, the detection limit in these latter experiments ranged from less than 1 transformant per 10^2 to 10^4 bacterial cells sampled. The addition of the antibiotic kanamycin in the drinking water of two of the rats did not yield observable transformants either. As seen from Table 1, the concentration of kanamycin added to the drinking water removed sensitive phenotypes.

Observing the discrepancy in the colonization ability of the *A. baylyi* strain between rats and mice, a 3rd experiment series was performed, this time adding the DNA 24 h prior to bacterial colonization of mice

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GITs. This was done to enable the possible uptake of DNA during bacterial colonization, a time period when *in vitro* investigations have shown that the *A. baylyi* strain used is highly competent. Considering that a lysed cell suspension of bacteria may be a better source of chromosomal DNA than purified DNA (Nielsen et al., 2000), two mice were also exposed to bacterial cell lysates rather than purified DNA. Despite the many changes in the parameters, *in vivo* uptake of DNA was not demonstrated in this mouse model. The CFU numbers in the different parts of the GI tract were lower than in experiment series 1, due to storage of the mice at −70 °C before dissection and sampling (Tab. 1).

To possibly increase the colonization level of *A. baylyi* cells in the GIT of mice, and hence improve the detection limit of possible transformants, co-inoculation with *Escherichia coli*, *Pseudomonas stutzeri* or *Lactobacillus rhamnosus* was attempted in experiment series 4. However, co-colonization did not increase the colonization level of *A. baylyi* in the GIT of mice. The probiotic *L. rhamnosus* strain LGG even reduced the number of CFUs of *A. baylyi* (Tab. 1).

Drawing on the knowledge obtained from the four experimental series described above, a long-term feeding experiment (experiment series 5) was initiated to examine the potential for bacterial uptake of DNA in monoassociated mice after continuous feeding with a DNA source for four weeks. Two mice were continuously given 10 $\mu g.mL^{-1}$ purified DNA from *A. baylyi* strain KTG, and the antibiotic kanamycin (0.4 $\mu g.mL^{-1}$) in the drinking water. The low concentration of kanamycin added was determined by identifying the concentration of the aminoglycoside antibiotic that led to a slight decrease in recipient cell numbers during *in vitro* growth, without being lethal to the larger population. The antibiotic was added to produce a slight, but stable fitness increase for potential transformants arising in the GIT. Twice a week, the two mice were also given suspension of *A. baylyi* strain BD413 (3×10^8 cells) to ensure stable bacterial colonization of the GIT. As seen in Table 1, transformants were not detected in any compartment of the GIT, sampled after a 4-week exposure period. Feces samples collected twice weekly were also sampled for the presence of potential transformants, but none were detected. In addition to direct plating of gut samples on selective media, enrichment of transformants was attempted by incubation of gut content in LB with 50 $\mu g.mL^{-1}$ kanamycin and 50 $\mu g.mL^{-1}$ rifampicin for 24 h, before plating. One colony from a small intestine sample was detected after enrichment. The possibility that this transformant arose in the enrichment step and not in the GIT cannot be excluded.

Transformation of *A. baylyi* with DNA present in recovered rodent gut contents

To assess whether feed-ingested DNA persists in the GIT of rodents in a form that makes it available to competent bacteria, rats with a normal microbiota were fed pellets with 50 μg purified DNA or bacterial cell lysates (containing DNA) added. The contents of the various GIT compartments were sampled and exposed to competent bacteria. The negative results of the transformation assays conducted, as well as positive and negative control treatments, are shown in Table 2.

Transformation of *A. baylyi* with freshly added DNA in the presence of gut contents

Gut content samples from various compartments of rats or mice were mixed with 1 μg purified DNA and an overnight culture of *A. baylyi* strain BD413 in LB medium. As seen from Table 3, transformation of *A. baylyi* was observed at low frequencies in the presence of content of the cecum and the large intestine of germfree mice. However, the presence of gut material from most sites sampled, including those from rats, resulted in sharply lowered transformation frequencies. In most cases, the transformants were not detectable (threshold of detection, 10^{-9}). In contrast, the absence of gut material in the transformation assay produced transformation frequencies of 10^{-3} (Tab. 3).

Growth of *A. baylyi* strain BD413 in different rodent feed sources

In the various experiments described above, the DNA administration was done with rodents receiving standardized feed (sterilized in most cases) as a part of their normal rearing and maintenance. Since the growth of the bacterial inoculum is mainly determined by the colon content and, hence, feed source, additional experiments were carried out to examine the growth of *A. baylyi* cells in different feed source suspensions. As seen in Figure 1, the most commonly used feed sources supported rapid growth of *A. baylyi* cells, although yielding somewhat differing growth dynamics when compared to LB media.

In vitro transformation assays of bacterial species frequently found in the GIT

Well-described bacterial isolates, including 15 bacterial type culture strains, obtained form the Japan Collection of Microorganisms, were examined for competence

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Table 2. Transformation of *A. baylyi* in gut contents obtained from rats fed DNA or cell lysate of *A. baylyi* KTG, or cell lysate from *E. coli* DHα-pKT-Km.

Transformation assay[a] in gut content of rats fed on							Controls[b]			
	DNA of KTG strain		Lysate of KTG strain		Lysate of DHα-pKT strain		One μg *KTG* DNA added		No added bacteria	
Time	Total CFU	TF	Total CFU	TF	Total CFU	TF	Total CFU	TF	Total CFU	TF
3 h										
Stomach	$1.8\ (0.3) \times 10^{10}$	$< 5.5 \times 10^{-11}$			$6.2\ (2.5) \times 10^{9}$	$< 1.6 \times 10^{-10}$	$3.1\ (1.8) \times 10^{9}$	2.0×10^{-5}	0	0
Small intestine	$5.0\ (1.5) \times 10^{9}$	$< 2.0 \times 10^{-10}$	$1.3\ (0.6) \times 10^{9}$	$< 7.6 \times 10^{-10}$	$3.6\ (2.5) \times 10^{9}$	$< 2.8 \times 10^{-10}$	$1.4\ (0.6) \times 10^{9}$	$< 7.2 \times 10^{-10}$	0	0
Cecum	$2.1\ (1.9) \times 10^{9}$	$< 4.8 \times 10^{-10}$	$6.3\ (1.9) \times 10^{9}$	$< 1.6 \times 10^{-10}$	$6.1\ (1.7) \times 10^{9}$	$< 1.6 \times 10^{-10}$	$5.9\ (2.4) \times 10^{9}$	$< 1.7 \times 10^{-10}$	0	0
Colon	$6.4\ (1.8) \times 10^{9}$	$< 1.6 \times 10^{-10}$	$6.9\ (2.1) \times 10^{9}$	$< 1.5 \times 10^{-10}$	$1.2\ (0.7) \times 10^{9}$	$< 8.5 \times 10^{-10}$	$6.2\ (2.7) \times 10^{9}$	$< 1.6 \times 10^{-10}$	0	0
6 h										
Stomach	$4.1\ (1.7) \times 10^{9}$	$< 2.4 \times 10^{-10}$	$1.7\ (1.0) \times 10^{10}$	$< 6.1 \times 10^{-11}$	$1.1\ (0.2) \times 10^{10}$	$< 9.1 \times 10^{-11}$				
Small intestine	$3.1\ (0.4) \times 10^{9}$	$< 3.2 \times 10^{-10}$	$1.8\ (0.9) \times 10^{9}$	$< 5.7 \times 10^{-10}$	$2.0\ (0.5) \times 10^{9}$	$< 5.1 \times 10^{-10}$				
Cecum	$1.7\ (0.6) \times 10^{9}$	$< 6.1 \times 10^{-10}$	$2.4\ (0.4) \times 10^{9}$	$< 4.1 \times 10^{-10}$	$1.2\ (1.1) \times 10^{9}$	$< 8.3 \times 10^{-10}$				
Colon	$1.5\ (0.4) \times 10^{9}$	$< 6.5 \times 10^{-10}$	$2.4\ (0.5) \times 10^{9}$	$< 4.1 \times 10^{-10}$	$2.1\ (1.1) \times 10^{9}$	$< 4.8 \times 10^{-10}$				
16 h										
Stomach	$4.1\ (1.7) \times 10^{9}$	$< 2.4 \times 10^{-10}$	$1.2\ (0.2) \times 10^{9}$	$< 8.9 \times 10^{-11}$	$8.2\ (8.7) \times 10^{9}$	$< 1.2 \times 10^{-10}$				
Small intestine	$3.1\ (0.4) \times 10^{9}$	$< 3.2 \times 10^{-10}$	$2.8\ (0.9) \times 10^{9}$	$< 3.6 \times 10^{-10}$	$7.7\ (6.8) \times 10^{9}$	$< 1.3 \times 10^{-10}$				
Cecum	$1.7\ (0.6) \times 10^{9}$	$< 6.1 \times 10^{-10}$	$9.1\ (8.0) \times 10^{9}$	$< 1.0 \times 10^{-10}$	$6.7\ (2.7) \times 10^{9}$	$< 1.5 \times 10^{-10}$				
Colon	$1.5\ (0.4) \times 10^{9}$	$< 6.4 \times 10^{-10}$	$3.3\ (0.7) \times 10^{9}$	$< 3.0 \times 10^{-10}$	$4.1\ (0.9) \times 10^{9}$	$< 2.4 \times 10^{-10}$				

[a] Transformation assays were performed by incubating 0.1 g gut content with 7×10^{6} cells of *A. baylyi* (20 μL) in 1 mL LB medium, with shaking, for 24 h.
[b] Positive transformation controls performed under the same conditions, with 1 μg strain *KTG* DNA, but without added gut content, yielded $1.0\ (\pm 0.3) \times 10^{9}$ total CFU and a transformation frequency of 7.8×10^{-3}.

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Table 3. Transformation assays of *A. baylyi* strain BD413 in LB-medium in the presence of contents sampled from various intestinal tract compartments from germfree mice or rats with normal microbiota.

	Germfree mice		Rats with normal microbiota		LB-medium only	
Gut sample added	Total CFU	TF[a]	Total CFU	TF	Total CFU	TF
None					$1.0 (\pm 0.3) \times 10^{9}$	7.8×10^{-3}
Stomach	N.d.[b]	N.d.	$3.1 (\pm 1.8) \times 10^{9}$	2.0×10^{-5}		
Small intestine	$7.1 (\pm 3.8) \times 10^{9}$	$< 1.4 \times 10^{-10}$	$1.4 (\pm 0.8) \times 10^{9}$	$< 7.2 \times 10^{-10}$		
Cecum	$2.9 (\pm 1.0) \times 10^{9}$	9.2×10^{-9}	$5.9 (\pm 2.4) \times 10^{9}$	$< 1.7 \times 10^{-10}$		
Large intestine	$1.2 (\pm 0.5) \times 10^{9}$	9.6×10^{-9}	$6.2 (\pm 2.7) \times 10^{9}$	$< 1.6 \times 10^{-10}$		

[a] TF: transformation frequency per 24 h transformation period.
[b] N.d.: not done.

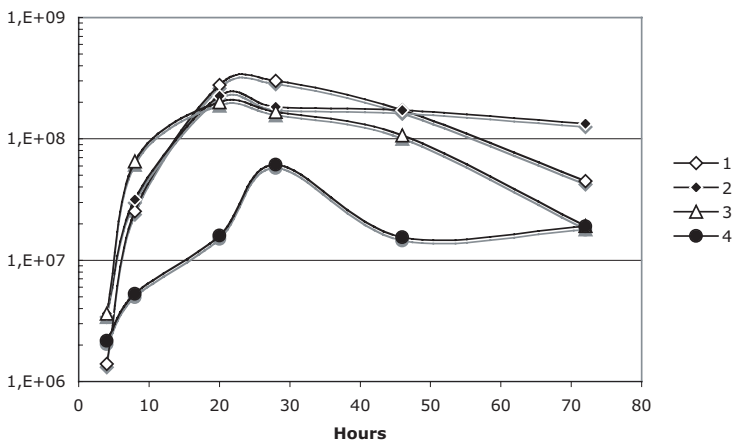

Figure 1. Growth dynamics (CFU.mL^{-1}) of *A. baylyi* strain BD413 in LB media and in different rat feed suspensions (1: LB media; 2: standard feed pellets, Animal Department, University of Tromsø, Norway; 3: standard feed pellets [R36, Lactamin], Animal Department, KI, Stockholm, Sweden; and 4: feed pellets [AIN-93M]).

for uptake of isogenic chromosomal DNA encoding rifampicin resistance. None of the isolates tested from the genera *Enterococcus, Streptococcus* and *Bifidobacterium* were found to be transformable during *in vitro* growth at levels above their spontaneous mutation rate to rifampicin (Tab. 4).

DISCUSSION

The lack of the experimental observance of HGT processes of free DNA in the GIT can be due to several factors: (i) true lack of competence-expressing bacterial cells in the GIT; (ii) lack of available DNA substrates at relevant concentrations in the GIT; (iii) a lack of a selective advantage of the horizontally-transferred DNA, so that rare bacterial transformants never surface in investigations working with limited gut sample sizes; (iv) a lack of sensitive studies that have examined the process with a reasonable sample size and detection limit; or (v) overall lack of suitable methods preventing an investigation of HGT processes relevant to bacterial evolutionary processes in the GIT (Nielsen and Daffonchio, 2007; Nordgård et al., 2005). Available information and methods do not allow a clear confirmation or elimination of any of the above explanatory models.

A series of experiments was designed and conducted to determine to what extent genetic transformation of the naturally competent bacterium *Acinetobacter baylyi* strain BD413 occurs in the GIT mice or rats. Bacterial growth (colonization) was hypothesized to be important for natural transformation to occur, since *A. baylyi* strain BD413 is known to develop competence during growth (Palmen and Hellingwerf 1997), both in nutrient-poor and nutrient-rich media (Nielsen et al., 1997). The colonization studies indicated that strain BD413 persists in the GIT in gnotobiotic rats at low levels, excluding this animal model for further studies. In gnotobiotic mice, colonization occurred at a comparably

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Table 4. Transformation assay of various bacterial strains exposed to homologous DNA (encoding Rif^R) and ratio of acquired Rif^R resistance in the presence or absence of DNA.

Strain and JCM accession number[a]	Source and accession year	Ratio of acquired resistance[b]
Enterococcus		
E. casseliflavus, JCM 5675	Plant, 1986	0.8
E. durans, JCM 8725^T	Dried milk, London UK, 1992	0.6
E. faecium, JCM 8714	Unknown, 1992	1.0
E. faecalis, JCM 8726^T	Unknown, 1992	0.7
E. faecalis, JCM 7783^T	Urine, 1990	1.2
E. gallinarum, JCM 8728^T	Chicken intestine, 1992	0.7
E. hirae, JCM 8718	Unknown, 1992	0.9
E. hirae, JCM 8720	Unknown, 1992	1.1
E. hirae, JCM 8719	Unknown, 1992	1.0
E. hirae, JCM 8729^T	Unknown, 1992	1.0
E. mundtii, JCM 8731^T	Soil, 1992	1.3
E. pseudoavium, JCM 8732^T	Bovine mastitis, 1992	0.8
E. raffinosus, JCM 8733^T	Blood culture, 1992	1.3
E. villorum, JCM 11557^T	Pig intestine, Canada, 2002	2.6
Streptococcus		
S. bovis, JCM 5346	Human blood, 1985	1.3
S. bovis, JCM 7902	Koala feces, 1991	1.0
S. equinus, JCM 7882	Equine abscess, 1991	0.8
S. equinus, JCM 7876	Cow dung, 1991	1.3
S. gallolyticus subsp. *macedonicus*, JCM 11119^T	Greek kasseri cheese, 2001	1.0
S. sp., JCM 5700	Unknown, 1986	1.1
Bifidobacterium		
B. angulatum, JCM 7096^T	Human feces, 1987	1.0
B. bifidum, JCM 1254	Intestine of adult, 1982	0.3
B. breve, JCM 1192^T	Intestine of infant, 1982	3.2
B. breve, JCM 1273	Intestine of infant, 1982	2.8
B. catenulatum, JCM 1194^T	Human feces, 1982	1.7
B. choerinum, JCM 1212^T	Pig feces, 1982	0.6
B. longum, JCM 7009	Infant feces, Japan, 1987	0.7
B. longum, JCM 7056	Infant feces, Japan, 1987	0.6
B. longum, JCM 7053	Infant feces, Japan, 1987	1.4
B. longum, JCM 7055	Infant feces, Japan, 1987	0.6
B. longum, JCM 7013	Infant feces, Japan, 1987	1.5
B. longum, JCM 7010	Infant feces, Japan, 1987	0.6
B. longum, JCM 1210	Intestine of infant, 1982	0.7
B. longum, JCM 1217^T	Intestine of adult, 1982	0.4
B. parvulorum, JCM 7022	Infant feces, Japan, 1987	1.2
B. pseudocatenulatum, JCM 7041	Human feces, Japan, 1987	0.5
B. pseudocatenulatum, JCM 7050	Human feces, Japan, 1987	1.4
Mean ratio ± SD		1.1 ± 0.6

[a] JCM: Japan Collection of Microorganisms; #: ^{T} = type strain.
[b] The ratio of acquired Rif^R resistance was calculated as the average frequency of Rif^R CFUs obtained with DNA treatment divided to the average frequency of Rif^R CFU obtained under the same cultivation conditions, but without added DNA.

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higher level, possibly due to improved oxygen delivery, due to the higher mucosal surface per unit content in mice. The overall numbers of CFU of bacteria colonizing the GIT and present in the extracted sample will determine the lower detection limit, because it is only possible to measure transformation frequencies that are higher than the reciprocal of the total numbers of bacterial cells/generations examined. Knowledge of the population dynamics and colonization pattern of the inoculum was therefore essential to guide subsequent transformation studies and data interpretation.

Numerous conditions were applied to facilitate high levels of bacterial colonization of the GIT, including co-colonization with other bacterial species (Tab. 1). Organic carbon sources (0.2% lactate) known to stimulate growth and transformation of *A. baylyi in vitro* (Nielsen et al., 1997) were also added to the inoculum to possibly improve the colonization level. Moreover, the growth of *A. baylyi* was investigated in various feed sources (Fig. 1). The feed source is of mutual and competitive interest to both the inoculated bacterium and the rodent host. It is therefore difficult to extrapolate the optimal feed source with respect to sustained growth of the inoculum at the *in situ* site of interest, as the feed nutrients will be released from the food and absorbed from the GIT at various locations and time points. The addition of a defined carbon source, such as lactate, to the feed to enhance bacterial growth rates is also non-straightforward since the GIT readily adsorbs most simple nutrient sources. Simple carbon sources added to rat feed may thus not reach the desired lumen locations where the specific bacterial inoculum establishes.

Various parts of the GIT support different bacterial densities (O'Hara and Shanahan, 2006). Although the *A. baylyi* strain was found at reasonable levels in the various gut compartments in mice (10^3–10^5 CFU per gram GIT content), such population levels are too low to provide insight into HGT processes occurring in the gut (see Nielsen and Townsend (2004) for a more in-depth discussion on detection limits of gene transfer in the GIT). Lower levels of gene transfer than those measurable experimentally in single mice models may be ecologically relevant (Pettersen et al., 2005). However, ethical, economic and practical considerations limits the number of mice that can be employed, thus, the focus in experimental design must be on how to extract as much information as possible from single animal models.

The *A. baylyi* strain is originally derived from soil and may not be adapted to the bile and partially anaerobic conditions in the GIT of mice. The main advantage using *A. baylyi* is the exceptional high level of competence achievable during normal growth. Selecting a bacterial inoculum with the ability to colonize at higher levels (*e.g.* 10^8 CFU per gram GIT content) will only improve the likelihood of detecting transformants if the competence level is equally high and easily inducible; however, most bacteria express competence less efficiently than *A. baylyi* (Lorenz and Wackernagel, 1994).

To further examine the role of gut content in preventing transformation of *A. baylyi* cells, contents from both normal microbiota rats, and germfree mice were added to *in vitro* transformation assays of competent cells of strain BD413 cells (Tab. 3). The presence of gut contents was inhibitory to transformation of *A. baylyi* under these conditions as well. Only purified DNA added to cecum and large intestine content samples from germfree mice was able to transform strain BD413 at low frequencies *in vitro*. Thus, this indicated that the strain may have the potential to become transformed *in vivo* at some sites in the GIT, given DNA is available at a sufficient quality and quantity. The sharply reduced DNA uptake frequencies also observed in the presence of sterile gut material from mice indicate that microbially produced DNA nucleases are not responsible for the absence of observable transformation. Bacterial nucleases have also in other studies been found to play a minor role in DNA degradation (Maturin and Curtiss, 1977; Wilcks et al., 2004). Wilcks et al. (2004) showed that plasmid DNA isolated from intestinal compartments from mono-associated rats can remain biological active (in electroporation assays of *E. coli*) after purification. Thus, a possible explanation for the sharp ~1 000 000-fold reduction of transformation frequencies in the presence of gut contents can be a reversible binding of DNA to feed surfaces and macromolecules that renders DNA inaccessible to competent bacteria. Further studies will be initiated to identify the causal factor behind the reduction in transformation frequency seen.

To our knowledge, no experimental studies have been able to demonstrate *in vivo* uptake of extracellular DNA in the GIT (with the exception of the mouth). This observation contrasts the studies reporting the ability of some bacterial species present in the GIT to become naturally transformed *in vitro* (Mercer et al., 1999a; Sun et al., 2006). Likely explanations for this non-uniform knowledge state include: (i) methodological limitations to the GIT sample size that can be practically analyzed for low frequency transfer events; (ii) a lack of understanding of ecological processes in the GIT limiting our ability to design biologically and tempo-spatially relevant HGT investigations; (iii) unidentified processes in the GIT that shields or adsorb DNA molecules during degradation process preventing measurable exposure to competent bacteria; or (iv) lack of competence development in naturally transformable bacteria in the GIT. It is concluded the frequently used model species for natural transformation, *A. baylyi*, is not detectably transformable in the GIT of rodents, and that available evidence does

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not suggest natural transformation to be a frequent event in the GIT. In future studies, careful consideration and optimization of the recipient inoculum level obtainable ($>10^7$ CFU.g^{-1}) in various parts of the gut is essential to achieve detection limits of informative value. Moreover, the strong inhibitory effect of the gut content seen on *in vitro* transformation of actively growing *A. baylyi* cells, should be examined in other bacterial gene transfer model systems, to determine if such inhibition is a general feature of GIT material.

MATERIALS AND METHODS

Preparation of DNA and bacterial cell lysates for the rodent feeding experiments

One colony of *A. baylyi* strain BD413 (spontaneous rifampicin resistant, and containing a chromosomally-inserted, selectable kanamycin resistance gene (*npt*II) named strain KTG (Nielsen et al., 1997) was inoculated in 3 mL LB medium supplemented with 50 μg.mL^{-1} rifampicin and 50 μg.mL^{-1} kanamycin (LB^{Rif50Kan50}) and incubated for 7 h with shaking at 32 °C. One mL of the starting culture was subsequently added to 500 mL LB^{Rif50Kan50}, and incubated at 32 °C overnight with shaking. Chromosomal DNA was isolated from the bacterial culture using the QIAGEN Genomic-tip 500/G and the concentration determined using a UV-spectrophotometer (BioRad). For preparation of bacterial cell lysates, overnight cultures of the KTG strain were centrifuged at 5000× *g* for 5 min, resuspended in distilled water, recentrifuged, and resuspended in water to the initial volume. One-mL aliquots were heat-treated at 80 °C for 15 min, and sterility of the heat-treated lysate was confirmed by plating 100 μL aliquots of undiluted lysate on LB agar-plates (Nielsen et al., 2000).

Colonization and transformation studies in gnotobiotic rodents

Gnotobiotic (germfree) NMRI mice and AGUS rats were bred at the Department of Medical Microbial Ecology, Karolinska Institute, Stockholm, Sweden. The contents of the animal stomach, small intestine, cecum and large intestine were collected separately, plated on the specific media with or without rifampicin and incubated at 37 °C for 46 h before CFU determination. All animal experiments have been approved by relevant ethical committees. Five series of experiments were conducted:

Experiment series 1. Two germfree mice were given 0.2 mL of an overnight culture of a kanamycin susceptible and rifampicin resistant *A. baylyi* strain BD413 by gavage feeding (3×10^8 cells per 0.2 mL). The animals were sacrificed after 46 h to determine the colonization level of the GIT.

Experiment series 2. Four germfree rats were given 0.2 mL of an overnight culture of *A. baylyi* strain BD413 by gavage feeding (3×10^8 cells per 0.2 mL). All four rats were subsequently given 50 μg purified DNA (from *A. baylyi* strain KTG) by gavage feeding 24 h after receiving the bacterial inoculum. Two of the rats (rats 3 and 4) also received kanamycin (10 μg.mL^{-1}) in the drinking water during the whole experiment. One additional rat did not receive the bacterial inoculum or DNA. The rats (including the control) were killed 24 h after receiving DNA.

Experiment series 3. Two germfree mice (mice 1 and 2) were given 10 μg.mL^{-1} purified DNA in the drinking water and two mice (mice 3 and 4) were given 10 μg.mL^{-1} lysed cells (of the same bacterial strain used for DNA isolation) in the drinking water. After 24 h, mouse 2 and 4 were given 0.2 mL of an overnight culture of *A. baylyi* strain BD413 by gavage feeding (3×10^8 cells per 0.2 mL). After another 24 h, all 4 mice were killed. One mouse did not receive bacteria or DNA and was used as a sterility control. An additional DNA-exposed mouse was killed after 48 h, rather than 24 h after receiving the bacterial inoculum (mouse 6).

Experiment series 4. For mixed colonization experiments, *A. baylyi* strain BD413, *E. coli*DH5-alfa, and *Pseudomonas stutzeri* strain JM303 were grown overnight at 37 °C in LB broth with shaking (220 rpm). For strain BD413, the broth was supplemented with 50 μg.mL^{-1} rifampicin. *Lactobacillus rhamnosus* (*LGG*), ATTC 53103, was grown in DeMan-Rogosa-Sharpe broth (MRS) overnight at 37 °C. Germ-free mice received a 6-mL aliquot of the above suspension (1.5×10^9 cells per mL) on the fur. Mouse 1 was given a suspension with the same amount of strains BD413 and strain LGG, mouse 2 strains BD413 and DH5-alfa, mouse 3 strains BD413 and JM303, and mouse 4 strain BD413 only. Mouse 5 was not exposed to any bacterial suspension.

Experiment series 5. The potential for bacterial uptake of DNA in mono-associated (*i.e.* containing only one bacterial species) mice after continual feeding with a DNA source for four weeks was investigated in two mice. The mice were given 10 μg.mL^{-1} purified DNA from *A. baylyi* strain KTG in the drinking water continually. In addition, 0.4 μg.mL^{-1} kanamycin was added to the drinking water as a weekly selection. Twice a week, the mice were also given a 0.2 mL suspension of *A. baylyi* strain BD413 (3×10^8 cells per 0.2 mL) in 0.2% lactate. After four weeks, the mice were killed and the different compartments of the GIT were collected to investigate the possible presence of *in vivo* transformants after plating on selective media. In addition to direct plating of the gut content, enrichment of transformants was attempted as follows: 0.1 g of the sample material of each of the GIT compartments was suspended in 1 mL LB LB^{Rif50Kan50} to select for potential transformants. The samples were

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incubated with shaking at 37 °C for 24 h before plating on selective media. Feces samples were also collected twice a week for four weeks. The feces samples were stored in LB medium with 20% glycerol at −70 °C before plating on selective media to identify possible transformants.

Determination of total CFU and transformant CFU in the GIT samples

One-hundred μL of ten-fold dilutions made in saline, of the content from the different sample sites (stomach, small intestine, cecum and large intestine) were plated on LB agar-plates supplemented with rifampicin (50 $\mu g.mL^{-1}$) for enumeration of total CFU of *A. baylyi* strain BD413 and on plates with rifampicin and kanamycin (both 50 $\mu g.mL^{-1}$) for transformant CFU determination. CFU were determined after incubation at 30 °C for 72 h. Calculated transformation frequencies are given as the number of CFU growing on transformant-selective plates divided by the number of CFU on recipient-selective plates. The detection limit is the reciprocal of the number of CFU on recipient-selective plates.

Transformation assay in recovered gut contents from rats fed DNA

To assess the possibility of foreign DNA to persist in the intestinal environment and whether surviving DNA remains biologically active, 11 Wistar rats (with a normal microbiota) were given feed pellets (AIN-93M) with 50 μg purified DNA added once, and then sacrificed at different time points. The control group consisted of two rats given no DNA; one was killed after 3 h and one after 6 h. These experiments were conducted at the Animal Department, University of Tromsø, Norway, and were approved under the auspices of the National Animal Research Authority. The rats were separated into different cages according to what kind of DNA treatment they were given (No DNA, chromosomal DNA from *A. baylyi* strain KTG, cell lysate from *A. baylyi* strain KTG, or lysate from strain *A. baylyi* DHα-pKT-Km). The preparation of purified DNA and cell lysate was performed as described above. The rats were killed after 3 h, 6 h and 16 h and the different compartments of the GIT were collected. The potential of the DNA present in the gut samples for transforming *A. baylyi* cells was assessed by mixing 0.1 g gut content with 20 μL of an overnight culture of *A. baylyi* strain BD413 in 1 mL LB medium. The solution was incubated at 37 °C for 24 h before plating on selective media to enumerate the total CFU and number of transformants. In addition, 1 μg purified DNA from *A. baylyi* strain KTG was mixed with 20 μL of an overnight culture of *A. baylyi* strain BD413 in 1 mL LB medium as a positive control.

Transformation of *A. baylyi* in LB media in the presence of rodent gut contents

To examine if the presence of gut content inhibited transformation, gut content (0.1 g) from various compartments of rats or mice was mixed with 1 μg purified DNA from *A. baylyi* strain KTG and 20 μL of an overnight culture of *A. baylyi* strain BD413 in 1 mL LB medium. The samples were incubated at 37 °C for 24 h with shaking before plating on selective media to enumerate total CFU and transformant CFUs.

Growth dynamics of *A. baylyi* in rodent feed sources and in LB medium with increasing kanamycin concentration

A. baylyi was grown in four different feed sources (LB media, standard rat feed used at the Animal Department, University of Tromsø, standard rat feed used at the Animal Department, Karolinska Institute, Stockholm, commercial rat feed preparation AIN-93M). The feed pellets were dissolved in water and the suspension was autoclaved. Twenty μL of an overnight culture of *A. baylyi* strain BD413 was added to 1 mL of each feed solution ($n = 3$). Samples were taken at different time points, diluted, plated in triplicates on selective media and incubated at 46 h at 32 °C before CFU enumeration. Feed suspensions with no added bacteria were used as negative controls.

A. baylyi strain BD413 was also grown in LB media with increasing concentrations of kanamycin (0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 $mg.L^{-1}$) to determine the lower concentration that affect growth of the inoculum. Twenty μL of an overnight culture of *A. baylyi* strain BD413 were added to 20 mL pre-heated LB^{Rif50}. Samples were taken at different time points (0, 4, 8, 24, 28 h) and the abs_{600} measured in triplicate on a Nanodrop spectrophotometer.

In vitro screening of natural transformation of various bacterial isolates

The Japan Collection of Microorganisms (JCM) generously provided all bacterial isolates studied. The media used and the growth conditions used were strain-specific and were selected as specified in the JCM strain catalogue (JCM, 2004). To select for spontaneous rifampicin resistant mutants (to be used as the DNA source in the subsequent transformation assay), rifampicin-sensitive colonies grown on solid media were inoculated in 1 mL of liquid media and incubated for 2 days in microcentrifuge tubes. A resuspended culture (200 μL) was transferred to solid media with rifampicin (20 $\mu g.mL^{-1}$) to

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select for resistant phenotypes. Single rifampicin resistant colonies were randomly picked, re-streaked for purity and inoculated on selective media. Genomic DNA was subsequently extracted from colonies obtained from two agar plates with freshly grown Rif^R cells using the QIAGEN QIAamp DNA stool mini Kit (Qiagen, Germany). The isolated DNA was quantified using a UV-spectrophotometer (Ultraspec 2100 PRO Amersham, England).

The transformation assay in liquid culture followed the method described by Ray and Nielsen (2005) with minor modifications. An overnight liquid culture of recipient cells was diluted in 500 µL PBS to approximately 10^6 CFU per mL. Aliquots of 100 µL were transferred into four microcentrifuge tubes and 1000 µL TS liquid broth was added. For each transformation assay, 40 µL of a DNA suspension with a minimum concentration of 30 $ng.\mu L^{-1}$ was added to two microcentrifuge tubes (the DNA used was the isogenic Rif^R counterpart to the specific strain examined). Forty µL PBS was added to the two other microcentrifuge tubes in the same assay, to quantify the occurrence of spontaneous Rif^R mutants. The tubes were then incubated in an aerobic incubator (or anaerobic jar in case of anaerobic species) at 37 °C for 24 to 48 h before plating in triplicate on media with (20 $mg.L^{-1}$) or without rifampicin. The plates were incubated at 37 °C until determination of CFUs. The following equation was used to quantify the ratio of acquired rifampicin among the tested strains: ratio = (A/B) divided to (C/D), where A = number of CFU growing on selective plates after exposure to isogenic DNA conferring Rif^R, B = total number of recipients CFU growing on unselective plates after exposure to isogenic DNA conferring Rif^R, C = number of CFU growing on selective plates without exposure to isogenic DNA, and D = total number of recipients CFU growing on non-selective plates without exposure to isogenic DNA.

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REFERENCES

Bensasson D, Boore JL, Nielsen KM (2004) Genes without frontiers. *Heredity* **92**: 483–489

Chambers PA, Duggan PS, Heritage J, Forbes MJ (2002) The fate of antibiotic resistance marker genes in transgenic plant feed material fed to chickens. *J. Antimicrob. Chemother*. **49**: 161–164

Chowdhury EH, Mikami O, Nakajima Y, Hino A, Kuribara H, Suga K, Hanazumi M, Yomemochi C (2003a) Detection of genetically modified maize DNA fragments in the intestinal contents of pigs fed StarLink™CBH351. *Vet. Hum. Toxicol*. **45**: 95–96

Chowdhury EH, Kuribara H, Hino A, Sultana P, Mikami O, Shimada N, Guruge KS, Saito M, Nakajama Y (2003b) Detection of corn intrinsic and recombinant DNA fragments and cry1Ab protein in the gastrointestinal contents of pigs fed genetically modified corn *Bt*11. *J. Anim. Sci.* **81**: 2546–2551

Chowdhury EH, Mikami O, Murata H, Sultana P, Shimada N, Yoshioka M, Guruge KS, Yamamoto S, Miyazaki S, Yamanaka N, Nakajima Y (2004) Fate of intrinsic and recombinant genes in calves fed genetically modified maize *Bt*11. *J. Food Protection* **67**: 365–370

Deaville ER, Maddison BC (2005) Detection of transgenic and endogenous plant DNA fragments in the blood, tissues, and digesta of broilers. *J. Agric. Food Chem.* **53**: 10268–10275

Doerfler W (2000) Foreign DNA in mammalian systems. Wiley-VCH, Weinheim, Germany

Duggan PS, Chambers PA, Heritage J, Forbes JM (2000) Survival of free DNA encoding antibiotic resistance from transgenic maize and the transformation activity of DNA in ovine saliva, ovine rumen fluid and silage effluent. *FEMS Microbiol. Lett.* **191**: 71–77

Duggan PS, Chambers PA, Heritage J, Forbes MJ (2003) Fate of genetically modified maize DNA in the oral cavity and rumen of sheep. *Br. J. Nutr.* **89**: 159–166

EFSA - European Food Safety Authority (2004) Opinion of the scientific panel on genetically modified organisms on the use of antibiotic resistance genes as marker genes in genetically modified plants. *EFSA J.* **48**: 1–18

Einspanier R, Klotz A, Kraft J, Aulrich K, Poser R, Schwägele F, Jahreis G, Flachowsky G (2001) The fate of forage plant DNA in farm animals: a collaborative case-study investigating cattle and chicken fed recombinant plant material. *Eur. Food Res. Technol.* **212**: 129–134

Forsman A, Ushameckis D, Bindra A, Yun Z, Blomberg J (2003) Uptake of amplifiable fragments of retrotransposon DNA from the human alimentary tract. *Mol. Gen. Genomics* **270**: 362–368

Hohlweg U, Doerfler W (2001) On the fate of plant or other foreign genes upon the uptake in food or after intramuscular injection in mice. *Mol. Gen. Genomics* **265**: 225–233

Jennings JC, Albee LD, Kolwyck DC, Surber JB, Taylor ML, Hartnell GF, Lirette RP, Glenn KC (2002) Attempts to detect transgenic and endogenous plant DNA and transgenic protein in muscle from broilers fed YieldGard corn borer corn. *Poultry Sci.* **82**: 371–380

https://doi.org/10.1051/ebr:2007029 Published online by Cambridge University Press

Kharazmi M, Sczesny S, Blaut M, Hammes WP, Hertel C (2003) Marker rescue studies on the transfer of recombinant DNA to *Streptococcus gordonii in vitro*, in foods and gnotobiotic rats. *Appl. Environ. Microbiol.* **69**: 6121–6127

Klotz A, Mayer J, Einspanier R (2002) Degradation and possible carry over of feed DNA monitored in pigs and poultry. *Eur. Food Res. Technol.* **214**: 271–275

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

Martín-Orúe SM, O'Donnell AG, Ariño J, Netherwood T, Gilbert HJ, Mathers JC (2002) Degradation of transgenic DNA from genetically modified soya and maize in human intestinal simulation. *Br. J. Nutr.* **87**: 533–542

Maturin L, Curtiss R (1977) Degradation of DNA by nucleases in the intestinal tract of rats. *Science* **196**: 216–218

Mercer DK, Melville CM, Scott KP, Flint HJ (1999a) Natural genetic transformation in the rumen bacterium *Streptococcus bovis* JB1. *FEMS Microbiol. Lett*. **179**: 485–490

Mercer DK, Scott KP, Bruce-Johnson WA, Glover AL, Flint HJ (1999b) Fate of free DNA and transformation of the oral bacterium *Streptococcus gordonii* DL1 by plasmid DNA in human saliva. *Appl. Environ. Microbiol.* **65**: 6–10

Mercer DK, Scott KP, Melville CM, Glover LA, Flint HJ (2001) Transformation of an oral bacterium via chromosomal integration of free DNA in the presence of human saliva. *FEMS Microbiol. Lett*. **200**: 163–167

Netherwood T, Bowden R, Harrison P, O'Donnell AG, Parker DS, Gilbert HJ (1999) Gene transfer in the gastrointetinal tract. *Appl. Environ. Microbiol.* **65**: 5139–5141

Netherwood T, Martín-Orúe SM, O'Donnell AG, Gockling S, Graham J, Mathers JC, Gilbert HJ (2004) Assessing the survival of transgenic plant DNA in the human gastrointestinal tract. *Nat. Biotechnol.* **22**: 204–209

Nielsen KM, Daffonchio D (2007) Unintended horizontal transfer of recombinant DNA. In Traavik T, Lim LC, eds, Biosafety first: holistic approaches to risk and uncertainty in genetic engineering and genetically modified organisms, Tapir Academic Press, Trondheim, Norway, pp 221–237

Nielsen KM, Townsend JP (2004) Monitoring and modeling horizontal gene transfer. *Nat. Biotechnol*. **22**: 1110–1114

Nielsen KM, Bones AM, van Elsas JD (1997) Induced natural transformation of *Acinetobacter calcoaceticus* in soil microcosms. *Appl. Environ. Microbiol*. **63**: 3972–77

Nielsen KM, Smalla K, van Elsas JD (2000) Natural transformation of *Acinetobacter* sp. strain BD413 with cell lysates of *Acinetobacter* sp., *Pseudomonas fluorescens* and *Burkholderia cepacia* in soil microcosms. *Appl. Environ. Microbiol.* **66**: 206–212

Nielsen KM, Berdal KG, Kruse H, Sundsfjord A, Mikalsen A, Yazdankhah S, Nes I (2005) An assessment of potential long-term health effects caused by antibiotic resistance marker genes in genetically modified organisms based on antibiotic usage and resistance patterns in Norway, VKM-Report, Norwegian Scientific Committee for Food Safety, Oslo, Norway

Nordgård L, Traavik T, Nielsen KM (2005) Nucleic acid isolation from ecological samples – vertebrate gut flora. In Methods in Enzymology, Academic Press, Vol. 224, pp 38–48

O'Hara AM, Shanahan F (2006) The gut flora as a forgotten organ. *EMBO Rep.* **7**: 688–693

Palka-Santini M, Schwarz-Herzke B, Hösel M, Renz D, Auerochs S, Brondke H, Doerfler W (2003) The gastrointestinal tract as a portal of entry for foreign macromolecules: fate of DNA and proteins. *Mol. Gen. Genomics.* **270**: 201–215

Palmen R, Hellingwerf KJ (1997) Uptake and processing of DNA by *Acinetobacter calcoaceticus*: A review. *Gene* **192**: 179–90

Pettersen AK, Primicero R, Bøhn T, Nielsen KM (2005) Modeling suggest frequency estimates are not informative for predicting the long-term effect of horizontal gene transfer in bacteria. *Environ. Biosafety Res.* **4**: 222–233

Ray JL, Nielsen KM (2005) Experimental methods for assaying natural transformation and inferring horizontal gene transfer. In Methods in Enzymology, Academic Press, Vol. 224, pp 491–520

Schubbert T, Lettmann C, Doerfler W (1994) Ingested foreign DNA (phage M13) DNA survives transiently in the gastrointestinal tract and enters the bloodstream of mice. *Mol. Gen. Genet.* **242**: 495–504

Schubbert R, Renz D, Schmitz B, Doerfler W (1997) Foreign (M13) DNA ingested by mice reaches peripheral leucocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. *Proc. Natl. Acad. Sci. USA* **94**: 961–966

Schubbert R, Hohlweg U, Renz D, Doerfler W (1998) On the fate of orally ingested foreign DNA in mice: chromosomal association and placental transmission to the fetus. *Mol. Gen. Genet.* **259**: 569–576

Sharma R, Alexander TW, John SJ, Forster RJ, McAllister TA (2004) Relative stability of transgene DNA fragments from GM rapeseed in mixed ruminal cultures. *Br. J. Nutr.* **91**: 673–681

Sharma R, Damgaard D, Alexander TW, Dugan MER, Aalhus JL, Stanford K, McAllister TA (2006) Detection of transgenic and endogenous plant DNA in digesta and tissues of sheep and pigs fed Roundup Ready Canola Meal. *J. Agric. Food Chem.* **54**: 1699–1709

Sun D, Zhang Y, Mei Y, Jiang H, Xie Z, Liu Z, Chen X, Shen P (2006) *Escherichia coli* is naturally transformable in a novel transformation system. *FEMS Microbiol. Lett.* **256**: 249–255

Thomas CM, Nielsen KM (2005) Mechanisms and barriers to horizontal gene transfer between bacteria. *Nat. Rev. Microbiol.* **3**: 711–721

Tony MA, Butschke A, Broll H, Grohmann L, Zagon J, Halle I, Dänike, Schauzu M, Hafez HM, Flachowsky G (2003) Safety assessment of *Bt*176 maize in broiler nutrition: degradation of maize-DNA and its metabolic fate. *Arch. Tierernahr.* **57**: 235–52

Wilcks A, Hoek AHAM, Joosten RG, Jacobsen BBL, Aarts HJM (2004) Persistence of DNA studied in different ex vivo and in vivo rat models simulating the human gut situation. *Food Chem. Toxicol.* **42**: 493–502

https://doi.org/10.1051/ebr:2007029 Published online by Cambridge University Press