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Thematic Issue on Horizontal Gene Transfer

An assessment of the potential of herbivorous insect gut bacteria to develop competence for natural transformation

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Whereas the capability of DNA uptake has been well established for numerous species and strains of bacteria grown *in vitro*, the broader distribution of natural transformability within bacterial communities remains largely unexplored. Here, we investigate the ability of bacterial isolates from the gut of grass grub larvae (*Costelytra zealandica* (White); Coleoptera: Scarabaeidae) to develop natural genetic competence *in vitro*. A total of 37 mostly species-divergent strains isolated from the gut of grass grub larvae were selected for spontaneous rifampicin-resistance. Genomic DNA was subsequently isolated from the resistant strains and exposed to sensitive strains grown individually using established filter transformation protocols. DNA isolated from wild-type strains was used as a control. None of the 37 isolates tested exhibited a frequency of conversion to rifampicin-resistance in the presence of DNA at rates that were significantly higher than the rate of spontaneous mutation to rifampicin-resistance in the presence of wild-type DNA (the limit of detection was approximately < 1 culturable transformant per 10^9 exposed bacteria). To further examine if conditions were conducive to bacterial DNA uptake in the grass grubs gut, we employed the competent bacterium *Acinetobacter baylyi* strain BD413 as a recipient species for *in vivo* studies. However, no transformants could be detected above the detection limit of 1 transformant per 10^3 cells, possibly due to low population density and limited growth of *A. baylyi* cells in grass grub guts. PCR analysis indicated that chromosomal *Acinetobacter* DNA remains detectable by PCR for up to 3 days after direct inoculation into the alimentary tract of grass grub larvae. Nevertheless, neither transforming activity of the DNA recovered from the alimentary tract of grass grubs larvae nor competence of bacterial cells recovered from inoculated larvae could be shown.

Keywords: natural transformation / New Zealand grass grub / *Acinetobacter* / DNA uptake / DNA persistence / GMO / biosafety

INTRODUCTION

Large-scale usage of genetically modified organisms (GMOs) in agriculture has raised concerns over the potential for transgenes to be horizontally acquired by representatives of various exposed microbial communities (Deni et al., 2005; Gebhard and Smalla, 1999; Nielsen et al., 1998; 2005; Paget et al., 1998). More than 10 different studies now show that if high DNA sequence similarity is present between the GMOs and the recipient bacterium, recombination of transgenes into the genome of naturally competent bacteria occurs at detectable frequencies *in vitro* (de Vries and Wackernagel, 1998; de Vries et al., 2001; 2004; Gebhard and Smalla, 1998; Nielsen et al., 2000b; Tepfer et al., 2003), in sterile soil (Nielsen et al., 2000b), or in infected tobacco plants (Kay et al., 2002a; 2002b). Without an introduced DNA sequence similarity, no studies have shown uptake of plant transgenes into exposed bacteria (Broer et al., 1996; Nielsen et al., 1997c; Schlüter et al., 1995). The extent to which natural homologies between transgenes and competent bacteria exist is unclear, but they may be prevalent, since most plant transgenes and vector sequences are modified from bacterial origins (Bensasson et al., 2004).

The conditions promoting competence development in many bacterial strains and species have been described (de Vries and Wackernagel, 2004; Lorenz and Wackernagel, 1994). However, few studies have determined the broader distribution of natural competence in a wider range of bacterial communities (Cohan et al., 1991; Sikorski et al., 2002; Stewart and Sinigalliano,

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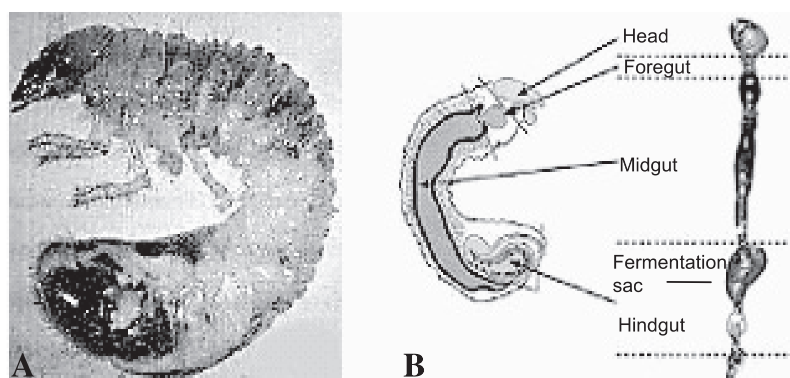

Figure 1. New Zealand grass grub (*Costelytra zealandica* (White)) (*Coleoptera: Scarabaeidae: Melolonthinae*). (a) Healthy specimen of the third instar larval stage. Note the darkly colored lumen contents beneath the white cuticle. (b) Dissected grass grub larval alimentary tract, showing the head, foregut, midgut, hindgut and fermentation sac. Figure adapted with permission from Hurst and Jackson (2002).

1990). Here we examine the transformability of a range of bacterial species obtained from within the gut of the herbivorous grass grub larva (*Costelytra zealandica*; Coleoptera: Scarabiaedae) from New Zealand. All of the 37 bacterial isolates characterized are thus representatives of an environment hypothesized to encounter exposure to nuclear and organelle DNA released from mechanically disrupted and ingested plant material in the insect gut (see review by Nielsen et al., this issue).

We also present more detailed studies of whether conditions are conducive for *in vivo* transformation of the *Acinetobacter baylyi* strain BD413 in the gut of the larvae of the New Zealand grass grub. Strain BD413 is naturally competent during growth, and has often been used for transformation studies *in vitro* (Averhoff et al., 1992; de Vries and Wackernagel, 2002; Juni, 1972; Juni and Janik, 1969; Palmen and Hellingwerf, 1997), in soil and water microcosms (Chamier et al., 1993; Clerc and Simonet, 1998; Lorenz et al., 1992; Nielsen and van Elsas, 2001; Nielsen et al., 1997a; 1997b), in a river (Williams et al., 1996), *in planta* (Kay et al., 2002a; 2002b; Tepfer et al., 2003) and most recently *in vivo* in tobacco horn worm (Deni et al., 2005). Strain DB413 was originally isolated from soil. Due to the high numbers of bacterial cells, an abundance of nutrients and constant supply of DNA from ingested food material and from the death of inhabiting microbes, the insect gut is an attractive location to study the potential for *in vivo* transformation (Deni et al., 2005; Mohr and Tebbe, 2006).

The New Zealand grass grub is an agricultural pest that feeds on roots of pasture plants during its larval stages. It is endemic to New Zealand but has adapted to feeding intensively on the roots of introduced pasture and crop plants (Ferro, 1976). The soil-dwelling larvae feed extensively on the grasses and clover in New Zealand's improved pastures, causing significant economic damage. At present there are no GM grasses or clover grown in the field in New Zealand, but GM varieties are under development both in New Zealand (McManus et al., 2005) and overseas (Wang and Ge, 2005). Larvae will also feed on the roots of a range of crops, including maize, carrots, strawberries, etc.

Larvae of the insect family *Scarabaeidae*, which includes the New Zealand grass grub, have a specialized compartment in the hindgut, referred to as the fermentation chamber, or sac. The fermentation sac (Fig. 1) is a key site of bacterial colonization in the alimentary tract of this insect (Chapman, 1985). Lumen contents are circulated within the fermentation sac for a period of time before passage into the rectum (Jackson et al., 1997). Recent research has focused on a natural pathogen of this insect, *Serratia entomophila* strain 154, which is the causative agent of amber disease in grass grub larvae. Amber disease is characterized by blockage of digestive enzyme secretion in the midgut, which in turn leads to cessation of feeding and ultimately death by starvation (Hurst et al., 2000). As digestion ceases, so does normal passage of lumen contents, and the fermentation sac becomes more or less sealed-off from both entry and exit, creating a kind of "incubator" for bacteria and nutrients present. In this study, we followed the colonization of directly inoculated BD413 cells in the alimentary tract of both healthy and *Serratia*-infected grass grubs, as well as the persistence of inoculated transforming DNA in the insect gut.

RESULTS

Genetic competence screen of insect gut isolates

Qualitative analysis of single-replicate filter transformations did not reveal any marked difference in the

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occurrence of rifampicin-resistance between the two treatments tested – exposure to wild-type DNA or Rif^R-encoding DNA – for the 37 grass grub larva gut bacterial isolates tested (Tab. 1). For quantitative analysis, we set an arbitrary cut-off ratio of 10 (transformant frequency/spontaneous mutation frequency) to warrant further investigation for transformability. None of the strains tested satisfied this criterion. The control strain *Pseudomonas stutzeri* JM303 always yielded a transformation/mutation ratio between 100 and 1000, with an average transformation frequency of about 10^{-6} transformants/recipient. Slightly higher transformation frequencies for the *P. stutzeri* strain have been reported earlier using chromosomal DNA in LB-agar transformations (Carlson et al., 1983).

Effect of insect gut lumen on growth and competence of *A. baylyi* strain BD413 *in vitro*

A. baylyi BD413 cells were tested both for their ability to grow in insect lumen contents and, subsequently, their ability to develop competence. When BD413 cells (one replicate per dissection sample) were exposed to lumen contents from freshly dissected grass grub alimentary tracts, BD413 numbers decreased from 1.8 ($\pm$ 0.2) $\times$ 10^8 to 7.7 ($\pm$ 1.0) $\times$ 10^6 cells, a 24-fold decrease, over 24 h. Moreover, when transforming DNA from *A. calcoaceticus* DSM586/*chr*::KTG (KTG) (Nielsen et al., 1997a) was included, no transformants were detected when assayed for kanamycin-resistant CFUs after 24 h (limit of detection $< 2 \times 10^{-7}$ transformants per recipients per 24 h). Bacteria in the positive control, BD413 cells exposed to LB media (without lumen content), grew and were competent for chromosomal DNA uptake (yielding approx. 1×10^{-4} transformants per recipient) for up to 48 h after inoculation. The negative control in which no DNA was added to BD413 cells in the presence of lumen content and LB medium yielded no kanamycin-resistant transformants, confirming also the absence of background kanamycin resistance in this system. Overall, these experiments demonstrate the poor growth and lack of genetic competence of BD413 in the presence of grub larva lumen content.

A. baylyi strain BD413 is a poor *in situ* colonizer of the grass grub gut

Recovery and enumeration of BD413 CFUs from alimentary tracts of orally-injected grass grub larvae indicated that BD413 was rapidly passed from the midgut to the fermentation sac (Tab. 2). BD413 cells could not be detected in the midgut or hindgut 72 h post-inoculation. High variability in colonization between individual grass grub larvae was observed, preventing statistical analysis as the variation was greater than the limit of detection. This could be due to inconsistencies in inoculation, as some grass grub larvae regurgitated a small amount of inoculum immediately after inoculation. The actual number of BD413 cells introduced into the alimentary tracts of each larva was therefore difficult to control. After 24 h, BD413 colonization of the midgut decreased about 250-fold, from 7600 to 30 CFU/midgut, and BD413 colonization of the hindgut decreased from about 85 CFU per hindgut to below the limit of detection in the same time period. After 24 h post-inoculation, BD413 was undetectable in both the midgut and the hindgut by cultivation-dependent methods. We were unable to recover BD413 cells from fecal pellets for all samples.

Colonization of the grass grub gut by *A. baylyi* strain BD413 when co-inoculated with the pathogen *Serratia entomophila* strain 154/pADAP

Prompted by the findings of Kay et al. (2002a), we tested whether *in vivo* colonization of BD413 would be more successful in the presence of an insect pathogen, *Serratia entomophila* strain 154, the causative agent of amber disease in New Zealand grass grub larvae. By inoculating BD413 cells into amber-diseased grass grub larvae, we wanted to test whether cessation of normal digestive passage would promote maintenance/survival of BD413 cells within the alimentary tract of grass grub larvae. As shown in Table 2, BD413 cells were detected in the alimentary tract of grass grubs up to 4 days after inoculation into *Serratia*-infected grubs. Also in this case, BD413 cells were passed rapidly through the midgut into the hindgut (fermentation sac), but maintained stable, albeit low, colonization levels for up to 4 days (end of detection period) after inoculation. After an initial inoculation dose of approximately 4.7×10^5 CFU into larvae that had been orally-injected with *Serratia* 24 h prior, we detected an average of 5.9×10^4 CFU of BD413 cells per midgut immediately after inoculation, with a 120-fold decrease after 24 h, and a further 16-fold decrease over the next 48 h, at which time colonization in the midgut stabilized at an average of < 100 CFU of BD413 cells per grass grub larva (Tab. 2). In the hindgut, however, BD413 cell numbers increased 480-fold during the first 24 h, from 13 to 6250 CFU $larva^{-1}$, after which colonization stabilized at about 1000–3000 CFU per hindgut up to 4 days post-inoculation. The presence of *Serratia* infection in grass grub larvae thus appears to stabilize both midgut and hindgut colonization by BD413. We were unable to detect BD413 in the faeces pellets sampled on days 3 and 4.

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Table 1. New Zealand grass grub larva (*Costelytra zealandica*) gut bacterial isolates tested for natural transformation *in vitro*.

Bacterial isolate[a]	% ID[b]	Accession[c] number	Frequency of Rif^R after exposure to Rif^R genomic DNA[d]	Frequency of Rif^R after exposure to wild type DNA[d]	Ratio (Rif^R/ Rif^S) frequencies[e]
***Acinetobacter* sp. (2)**	100	AM491044	$4.8 \times 10^{-8} - 1.1 \times 10^{-7}$	$5.1 \times 10^{-8} - 8.4 \times 10^{-8}$	0.9–1.3
Acinetobacter sp.	100	AM491045	3.9×10^{-9}	1.5×10^{-9}	2.5
Carnobacterium sp.	100	AM491046	1.3×10^{-7}	1.6×10^{-7}	0.9
Carnobacterium sp.	100	AM491046	1.3×10^{-7}	1.2×10^{-7}	1.1
Carnobacterium sp.	100	AM491046	1.1×10^{-7}	3.3×10^{-7}	0.3
Chryseobacterium sp.	99	AM491047	2.1×10^{-7}	2.5×10^{-7}	0.8
Enterobacteriaceae	100	AM491048	1.1×10^{-7}	3.3×10^{-7}	0.3
Erwinia sp.	100	AM491049	3.6×10^{-7}	8.6×10^{-7}	0.4
Erwinia sp.	99	AM491050	7.5×10^{-8}	7.4×10^{-8}	1.0
***Hafnia alvei* (2)**	100	AM491052	$1.2 \times 10^{-6} - 2.0 \times 10^{-6}$	$1.2 \times 10^{-6} - 2.0 \times 10^{-6}$	1.0–1.1
***Morganella* sp. (2)**	99	AM491051	$2.1 \times 10^{-6} - 8.9 \times 10^{-6}$	$2.8 \times 10^{-6} - 3.7 \times 10^{-6}$	0.8–2.4
***Ochrobactrum* sp. (2)**	100	AM491053	$1.2 \times 10^{-7} - 3.4 \times 10^{-7}$	$1.5 \times 10^{-7} - 5.1 \times 10^{-7}$	0.7–0.8
Ombesumbacterium proteus	99	AM491054	3.6×10^{-6}	3.3×10^{-6}	1.1
Pectobacterium carotovorum	99	AM491055	1.4×10^{-7}	1.8×10^{-7}	0.8
Pectobacterium carotovorum	98	AM491056	1.1×10^{-4}	8.5×10^{-5}	1.2
***Pectobacterium carotovorum* (2)**	98	AM491056	$1.6 \times 10^{-7} - 5.5 \times 10^{-5}$	$1.5 \times 10^{-7} - 6.2 \times 10^{-5}$	0.9–1.0
Providencia sp.	98	AM491057	8.8×10^{-8}	5.9×10^{-8}	1.5
Pseudomonas sp.	100	AM491058	3.3×10^{-7}	3.0×10^{-7}	1.1
Pseudomonas sp.	100	AM491059	2.8×10^{-7}	5.2×10^{-7}	0.5
Pseudomonas sp.	100	AM491060	9.7×10^{-8}	9.1×10^{-7}	1.1
Pseudomonas sp.	100	AM491061	1.3×10^{-7}	1.0×10^{-7}	1.2
Rhodococcus sp.	100	AM491062	5.6×10^{-8}	5.4×10^{-8}	1.0
***Rhodococcus* sp. (6)**	100	AM491062	$7.0 \times 10^{-8} - 3.5 \times 10^{-7}$	$6.9 \times 10^{-8} - 3.5 \times 10^{-7}$	0.7–1.4
***Serratia* sp.** group 1a **(4)**	99	AM491063	$6.8 \times 10^{-8} - 6.1 \times 10^{-7}$	$1.5 \times 10^{-7} - 6.8 \times 10^{-7}$	0.5–1.2
***Serratia* sp.** group 1b **(2)**	99	AM491063	$2.3 \times 10^{-7} - 5.6 \times 10^{-7}$	$5.3 \times 10^{-7} - 6.4 \times 10^{-7}$	0.4–0.9
Serratia sp. group 1c	99	AM491063	4.0×10^{-7}	1.4×10^{-6}	0.3
***Serratia* sp.** group 2a **(3)**	99	AM491064	$7.3 \times 10^{-9} - 9.4 \times 10^{-8}$	$8.3 \times 10^{-9} - 9.7 \times 10^{-8}$	0.8–1.0
***Serratia* sp.** group 2b **(2)**	99	AM491064	$3.3 \times 10^{-8} - 8.3 \times 10^{-8}$	$3.3 \times 10^{-8} - 7.7 \times 10^{-8}$	1.0–1.1
Serratia sp. group 2c	99	AM491064	1.4×10^{-7}	8.5×10^{-8}	1.7
***Serratia* sp.** group 2d **(2)**	99	AM491064	$5.2 \times 10^{-8} - 1.9 \times 10^{-7}$	$5.8 \times 10^{-8} - 1.8 \times 10^{-7}$	0.9–1.1
Serratia sp.	99	AM491065	3.0×10^{-7}	5.6×10^{-8}	5.3
Serratia sp.	99	AM491066	1.4×10^{-7}	1.3×10^{-7}	1.1
Serratia sp.	99	AM491067	9.7×10^{-8}	1.2×10^{-7}	0.8
***Staphylococcus pasteuri* (2)**	100	AM491068	$3.0 \times 10^{-7} - 1.2 \times 10^{-6}$	$3.6 \times 10^{-7} - 1.2 \times 10^{-6}$	0.8–1.0
Stenotrophomonas sp.	100	AM491069	5.1×10^{-8}	5.5×10^{-8}	0.9
Yersinia frederiksenii	99	AM491043	1.2×10^{-7}	5.2×10^{-8}	2.3
***Yersinia frederiksenii* (6)**	99	AM491043	$3.9 \times 10^{-8} - 5.1 \times 10^{-7}$	$4.0 \times 10^{-8} - 5.1 \times 10^{-7}$	0.3–2.2
Pseudomonas stutzeri[f]	100	AM491070	$3.9 (\pm 3.2) \times 10^{-5}$	$5.2 (\pm 5.1) \times 10^{-8}$	630

[a] 16S rRNA partial sequences were aligned using BLAST (default parameters) and the Ribosomal Database Project II (http://rdp.cme.msu.edu). Isolates with 100% similarity to each other at the 16S rDNA locus within each group were tested by RAPD-PCR and BOX-PCR to confirm/reject clonality. Divergent banding patterns (⩾1 band difference) were designated as different strains and included in the table as separate isolates. For *Serratia* spp., two separate groupings with 100% internal similarity were identified, group 1 and group 2. Subgroup designations (1a–1c and 2a–2d) were assigned based on unique BOX-PCR banding patterns (data not shown).

[b] Percent identity (% ID) of partial 16S rRNA genes to closest BLAST hit in the bacterial databases.

[c] Accession numbers are for partial 16S rRNA sequence for each isolate.

[d] Natural transformation assays (single replicates) of various grass grub larval isolates *in vitro* in the presence of wild-type or rifampicin-resistant (Rif^R) genomic DNA. Where multiple clones of the same isolate were tested (number in brackets), the range of transformation frequencies obtained using all clones for that isolate is shown.

[e] Ratio of (Frequency rifampicin resistance after exposure to Rif^R DNA/Frequency of Rif^R after exposure to wild type DNA). The range of ratios is given when isolates were represented by more than one BOX-PCR indistinguishable clone.

[f] Transformation frequencies for *P. stutzeri* JM303 are shown as average (± SD), and represent the average transformation frequency from all experiments were JM303 was used as positive control.

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Table 2. Colonization of the gut of New Zealand grass grub larvae by *Acinetobacter baylyi* strain BD413 (pooled sampling), and *in vitro* transformation screen of BD413 cells recovered from the gut content.

Treatment / Sampling	*n*[a]	Midgut CFU[a]	Hindgut CFU[a]	Transformation frequency[b]
Healthy larvae				
Inoculum (CFU larva^{-1})		2.1×10^5		
Sampling day 0	4	7.6×10^3	8.5×10^1	n.d.
day 1	4	3.0×10^1	< 5	n.d.
day 2	4	< 5	< 5	n.d.
day 3	4	< 5	< 5	$< 9.3 \times 10^{-8}$
day 4	3	< 5	< 5	n.d.
Serratia-infected larva[c]				
Inoculum (CFU larva^{-1})		4.8×10^5		
Sampling day 0	4	5.9×10^4	1.3×10^1	n.d.
day 1	4	4.9×10^2	6.3×10^3	$< 2.3 (\pm 1.8) \times 10^{-9}$
day 2	4	1.1×10^2	1.5×10^3	n.d.
day 3	4	< 5	2.2×10^3	$< 1.1 (\pm 1.0) \times 10^{-8}$
day 4	4	3.0×10^1	2.8×10^3	$< 6.4 (\pm 1.1) \times 10^{-8}$

[a] Average CFU counts were calculated by dividing the CFU count by *n* number of larvae sampled for each time point. Limit of detection was 1 CFU per 100 μL plated macerate suspension, or 5 CFU per 500 μL total macerate suspension. Samples in which no CFUs were recovered are shown as < 5 CFU *per pooled sample*.

[b] Transformation frequencies of BD413 cells recovered from freshly dissected insect lumen contents with *A. baylyi* DSM586/*chr*::KTG chromosomal DNA. n.d., no BD413 cells were recovered from the 24-h transformation reactions. Where BD413 cells were recovered after the transformation period, but no transformants were detected, the limit of detection is given. Transformation frequency = transformants/recipient ± standard deviation. Positive controls consisted of 10^6 BD413 cells and 1 μg KTG DNA in 500 μL LB broth only (*i.e.* BD413 cells were not exposed to insect lumen content before or during transformation) and yielded frequencies of $1.1 (\pm 0.7) \times 10^{-4}$.

[c] *Serratia* co-infection was verified by plating gut macerates on CTA agar (selective for *Serratia*) and incubating at 30 °C for 3–5 days. All *Serratia* co-infected grass grub larvae tested positive for *Serratia* colonization.

A. baylyi strain BD413 cells are not competent for DNA uptake immediately after recovery from the insect gut

Liquid culture transformation assays were performed on gut-recovered BD413 cells by exposing 50 μL freshly dissected lumen content (one replicate per dissection sample) from BD413-inoculated grass grub larvae to a high concentration of purified homologous chromosomal DNA (Tab. 2). After incubation for 24 h at 33 °C, cells were pelleted by centrifugation and appropriate dilutions were plated on LB amended with 50 μg.mL^{-1} rifampicin and 50 μg.mL^{-1} cycloheximide (LBRC) (to enumerate total BD413 cells) and LBRC agar amended with kanamycin (LBRCK). Despite the enrichment for BD413 cells during the 24-h transformation period in media amended with rifampicin (to eliminate normal gut microflora), we were unable to detect transformants by cultivation-dependent methods. The limit of detection for transformation for those experiments in which BD413 cells were recovered varied from 10^{-7} to 10^{-9}. Control samples in which competent BD413 cells were incubated with KTG DNA in LB broth (*i.e.* in the absence of insect lumen content) always yielded transformants at a frequency of about 10^{-4}, in accordance with previous studies (Nielsen et al., 1997a; 1997b).

The expected high pH of insect lumen contents, approximately pH 10 in the midgut and pH 8 in the hindgut (Biggs and McGregor, 1996), could reduce the ability of BD413 cells either to grow or to maintain competence in the insect gut. Using pH strips, the pH of grass grub larvae lumen content was found to be around 7.0 in the midgut and about 8.0 in the hindgut (data not shown). Disruption of the lumen could, in addition to the lack of precision in the measurements using pH strips, have affected the measured pH values. To further determine the effect of pH on growth and competence of BD413 cells, we cultured ~5×10^6 stationary phase BD413 cells with 500 ng KTG DNA in LB media with pH values ranging from 7.0–12.0 in one-pH-step increments and took single measurements of growth at 6, 24, and 48 h after inoculation (Andersen, 2005). We observed an increase in growth over the 48-h experimental period in all cultures except those at pH 11.0 and 12.0, in which cell numbers had decreased to below the level of detection at 48 h. Kanamycin-resistant transformants were detected

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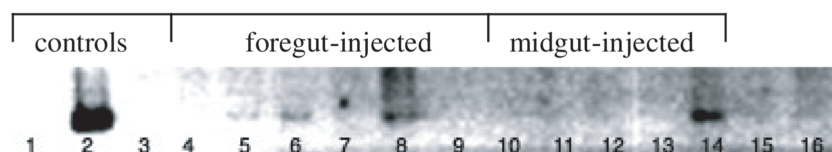

Figure 2. Detection of KTG in total DNA isolated from grass grub larva lumen content by PCR using primers IB3/4 (410 bp product) and 100 ng total DNA as template. **Lane 1**, negative water control. **Lane 2**, 10 ng KTG DNA. **Lane 3**, uninjected control larva 2 h. **Lanes 4–9**: foregut-injected. **Lane 4**, 2 h. **Lane 5**, 4 h. **Lane 6**, 24 h. **Lane 7**, 47 h. **Lane 8**, healthy larva 71 h. **Lane 9**, diseased larva 71 h. **Lanes 10–16**: midgut-injected. **Lane 10**, 2 h. **Lane 11**, 4 h. **Lane 12**, 24 h. **Lane 13**, 47 h. **Lane 14**, healthy larva 71 h. **Lane 15**, diseased larva 71 h. **Lane 16**, faeces pellets 47 h. PCR samples were separated on an agarose gel and stained with ethidium bromide. The original gel picture was inverted and adjusted in Photoshop Elements vers. 2.0 (Adobe Systems Inc., San Jose, California) to demonstrate PCR products more clearly.

at all three time points for pH values 7.0, 8.0 and 9.0. For pH 10, transformants were detected 24 h after inoculation (45-fold cell growth increase), but not at 6 h or 48 h post-inoculation. No transformants were detected at any time points at pH 11.0 and pH 12.0 (data not shown).

Added DNA can be detected by PCR for up to 3 days after injection into the insect gut

We used KTG- and *nptII*-specific conventional PCR to verify KTG DNA integrity over time in the lumen of individual healthy grass grub larvae that had received a 5 μL (approx. 3.9 μg) dose of KTG DNA by oral injection either into the foregut or the midgut. Using 100 ng total DNA isolated from freshly dissected larval lumen content as template, PCR products (410 bp) could be detected at up to 71 h after direct injection of DNA into either the foregut or the midgut (Fig. 2, lanes 4–14). The injection site of chromosomal bacterial DNA (*i.e.* into the foregut or midgut) appeared to have little influence on the stability of DNA in the alimentary tract. Two of the grass grubs originally injected with KTG DNA developed signs of amber disease over the course of the experiment. These grubs, one of which had been injected into the foregut and one of which had been injected into the midgut, were sampled at 71 h after inoculation and included in the PCR analysis. We were unable to detect the presence of KTG DNA in this sample (Fig. 2, lane 15), indicating that the health of the grass grub larvae may affect the stability of DNA in the alimentary tract. KTG DNA was not detected in faecal pellets sampled at 47 h post-inoculation (Fig. 2, lane 16).

To determine whether the total DNA extracted from freshly dissected lumen content retained transforming activity, we used 10 μL (approx. 1 μg) of each DNA preparation to transform (single replicates) 10^8 competent BD413 cells in liquid culture. After incubation for 24 h, no transformants were obtained for any of the reactions (transformation frequency $< 1.1\ [\pm 0.2] \times 10^{-8}$).

DISCUSSION

Genetic competence screen

The prospect of identifying competent strains from amongst naturally occurring bacterial communities is important for understanding the capacity of these strains to routinely access DNA in their natural habitats. The goal of the first part of this study was to develop an efficient method to screen bacterial isolates for natural competence. Many factors must be taken into consideration when designing and drawing conclusions from such an experiment, as outlined in Ray and Nielsen (2005). First and arguably most important, all strains, despite taxonomic diversity, were treated under homogeneous conditions. These conditions may have ignored variations in factors such as temperature, nutrient availability, stress or change of growth conditions, timing of DNA exposure, etc., that would be important for some individual strains to express competence (Matsui et al., 2003). In the absence of knowledge of the specific inducing factors, these cannot be mimicked within reasonable time and costs. Yet, as our screen was intended to identify competent species, further examination of the quantitative parameters enhancing competence would have been performed if positive results were obtained, *i.e.* transformation frequencies 10-fold above the mutation rate in a population of 10^8–10^9 bacterial cells.

Filter transformations, in which bacteria form biofilms on nitrocellulose membranes, are advantageous for large-scale screening of bacterial competence for several reasons. First, it is easy to control for growth and contamination of each individual strain. Second, the diluted cell suspensions spotted onto the filter enter a second log-phase during growth on the new media source (*i.e.* absorbed through the filter). This provides a spectrum of growth phases, during any of which the cells would be exposed to transforming DNA that could be taken up and expressed (provided that the DNA was not degraded early during the incubation period). The use of rifampicin-resistance as a selection marker is advantageous, because

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of the ease with which rifampicin-resistant mutants are generated and distinguished from actual transformation events. The wild-type DNA and saline controls were included as treatments for each strain to determine (a) the normal frequency of spontaneous rifampicin-resistance mutation in each strain as well as (b) any mutagenic effects that the physical presence of DNA (or contaminants in the DNA extract) may have on the cells, possibly increasing the spontaneous mutation rate to high enough levels that they would appear to be significantly different from the saline control.

Broad host range plasmid DNA was originally considered as a donor DNA source, since it precludes the necessity to generate genetically-marked individual donor DNA sources and perform numerous individual DNA isolations. Our initial experiments, however, did not yield positive results, even when an IncQ plasmid (pSKTG) (Smit and van Elsas, 1992), was used as donor DNA (data not shown). The reduced uptake efficiency of plasmids as compared to homologous chromosomal DNA, uncertainty related to the expression of the selectable trait and plasmid-host compatibility makes plasmid uptake assays less attractive. The use of heat-inactivated cell lysates as a source of transforming donor DNA (Nielsen et al., 2000a) was considered as an alternative to using purified genomic DNA, however, it is problematic to produce sterile lysates without compromising DNA quality, since different bacterial species differ in their heat-tolerance. In addition, we would have excluded all spore-forming bacteria from the screen.

Despite the numerous methodological advantages and limitations as discussed above, we can conclude that none of the 37 divergent strains and insect gut representatives tested provided any indications that they were transformable under the conditions provided. This suggests that none of the most dominant culturable species present in the grass grub larvae are highly transformable during rapid growth within frequency-ranges most often observed for other naturally transformable bacteria (de Vries and Wackernagel, 2004).

The insect larval gut represents an interesting possibility for testing the development of competence of insect-associated bacteria. We attempted to establish a model *in vivo* transformation system in the gut of larvae of New Zealand grass grub larvae using *A. baylyi* strain BD413 as recipient and purified chromosomal DNA from the isogenic strain *A. baylyi* strain DSM586/*chr*::KTG (Nielsen et al., 1997c) as donor DNA. We initially intended to use a natural insect gut isolate as the recipient, however, the earlier screen of the 37 grass grub gut isolates did not yield any naturally competent strains upon which we could focus for *in vivo* transformation. Many physiological parameters are important for the achievement of genetic competence and detection of transformation *in vivo*. To this end, we tested the growth and competence development of recipient BD413 in insect lumen contents *in vitro*, the effect of alkaline pH on growth and competence of BD413 *in vitro*, optimal method for delivery of BD413 cells and transforming KTG DNA into the gut of grass grubs, and the presence of transforming DNA *in vivo*.

Our results indicate that the alimentary tract of the insect species used was not conducive to growth, and thus competence development, of *A. baylyi* strain BD413. Our *in vitro* tests suggest also that nutrient limitation is not likely to be the main reason for lack of growth, as spiking lumen contents with LB did not restore growth to BD413 cells. For many of the transformation reactions with lumen-recovered BD413 cells, we were unable to recover BD413, even after 24 h incubation in optimal laboratory conditions. Thus, possible inhibitory factors or conditions present in the BD413-inoculated larval lumen content reduced inoculum growth and competence development. The observed increase in persistence of BD413 cells seen in *Serratia*-infected larvae could either indicate a release of growth-promoting nutrients, change in bacterial community composition, or blockage of growth-inhibitors. We tested the growth of BD413 in *Serratia*-infected grass grub larvae, but did not test whether the presence of *Serratia* in lumen contents was permissive for BD413 growth *in vitro*. *Serratia* infection results in blockage of peptidase production in the midgut. Proteases and other normal digestive enzymes are obvious potential growth-inhibitors for BD413 cells. On the other hand, the detection of transformation in *Serratia*-infected larvae would remain unlikely, given the poor growth of BD413 in the insect lumen (maximum 10^3 CFU.hindgut^{-1}). This level of colonization leads to assumption of a transformation frequency of at least 1 transformant per 10^3 recipient cells, a frequency rarely observed even under highly optimized *in vitro* conditions (Nielsen et al., 1997a). The single-species bacterial population size (up to approx. 10^3) present (or obtainable) in a single insect larva suggests small insect models are unsuitable to quantify horizontal gene transfer frequencies. Hypothesizing a gene transfer frequency of 10^{-8} per 24 h, approximately 10 000 single grass grub guts would have to be examined to sample such an amount of bacteria, an unrealistic task. Thus, given current limitations in the population sizes of single bacterial species (DNA recipients), HGT studies in insects are currently only informative to identify specific factors or conditions extremely conducive or preventive of natural transformation processes (see Nielsen and Townsend, 2004).

We found evidence that DNA is stable in the alimentary tract of healthy grass grubs for up to 3 days after direct injection into the midgut as shown by positive PCR for the bacterial chromosomal KTG sequence. More

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Table 3. PCR primers used in this study.

Primer	5'-3' sequence	Reference
16S27F	AGAGTTTGATCCTGGCTCAG	Lane, 1991
16S926F	AAACT(CT)AAA(GT)GAATTGACGG	Lane, 1991
16S1494R	CTACGGCTACCTTGTTACGA	Daffonchio et al., 1999
M13	GAGGGTGGCGGTTCT	Huey and Hall, 1989
BOXA1R	CTACGGCAAGGCGACGCTGACG	Versalovic et al., 1994
IB3	TCTCATGCTGGAGTTCTTCG	Nielsen et al., 1997c
IB4	CTGCTGTTCTATAGGACTGG	Nielsen et al., 1997c
NPT II Conner-F	ATGACTGGGCACAACAGACAATCGGCTGCT	Davidson et al., 2004
NPT II Conner-R	CGGGTAGCCAACGCTATGTCCTGATAGCGG	Davidson et al., 2004

sensitive (and quantitative) detection methods, such as hybridization or Real-Time PCR, might have improved our ability to detect and quantify DNA persistence in the insect gut. Our *in vitro* studies of KTG DNA stability in lumen contents suggest that DNA does not retain its transforming activity after injection into the insect alimentary tract at all time points tested.

For future studies, we would choose a natural recipient/host/selection system, and preferably an herbivorous host with a much larger larval stage to facilitate easier dissection of the alimentary tract. Moreover, a larger gut system would be preferable, given that the total bacterial population per gut system defines and limits the lower level of the transformation frequencies that can be practically measured per larva. It is thus necessary to consider the overall bacterial population size and accommodate sampling of a high number of insect guts, and possibly include *in situ* detection methods, for the experiments to be biologically meaningful.

MATERIALS AND METHODS

Bacterial strains

Unless otherwise stated, all strains were stored at –70 °C and cultured at 28 °C in Luria-Bertani (LB) broth, or LB supplemented with 10 $g.L^{-1}$ agar (LB agar). Bacterial strains (Tab. 1) were isolated from the gut contents of the New Zealand grass grub, *Costelytra zealandica* (White) (Coleoptera: Scarabiaedae), by plating the macerates of alimentary tracts that had been aseptically dissected from living specimens onto Tryptic Soy (TS) agar plates and incubation for several days at 28 °C. Individual colonies were randomly picked, streaked to purity on Mueller-Hinton (MH) agar and archived. Strains were Gram-stained before identification of Gram negative isolates using API 20E or API 20NE (bioMérieux Inc., Auckland, New Zealand) bacterial typing systems. Approximately 80 isolates were tested to determine their level of sensitivity to kanamycin and rifampicin, using Etest from AB Biodisk (Solna, Sweden). A total of 37 isolates with a minimum inhibitory concentration of < 4 $\mu g.mL^{-1}$ for either kanamycin or rifampicin were chosen for further investigation of their ability to develop natural genetic competence *in vitro*.

Identification of bacterial isolates by 16S genotyping

Approximately 100 ng chromosomal DNA (see below) from each isolate was used as template in 20 µL PCR reactions to amplify the 16S rRNA gene using 10 µL QIAGEN HotStar master mix (QIAGEN, Hilden, Germany) and 10 pmol each of primers 27F and 1494R (Tab. 3) with the following parameters: 95 °C/15 min, 30 cycles of 94 °C/1 min, 55 °C/1 min, 72 °C/1.5 min, followed by 72 °C/10 min and 4 °C until sample analysis. After verification of a single product by agarose gel electrophoresis, 5 µL of each PCR product was treated with 1 unit each of Antarctic phosphatase (New England Biolabs, Ipswich, Massachusetts) and Exonuclease I (USB, Cleveland, Ohio) using the following temperature program: 37 °C for 30 min, 80 °C for 15 min, and –20 °C until sequencing. Sequencing reactions were performed using Big Dye v3.1 cycle sequencing terminator reactions (Applied Biosystems, Foster City, California) according to the manufacturer's recommendations. Samples were analyzed on a 3130xl Genetic Analyzer (Applied Biosystems) and sequence traces were viewed and edited in Sequencer version 4.2.2 (Gene Codes, Ann Arbor, Michigan). Approximately 535 bp of each 16S sequence was compared to the bacterial databases using the *tblastn* algorithm (Altschul et al., 1997) and default parameters. Identity of each isolate was determined based on the highest scoring BLAST hit as well as the Ribosomal Database Project II (http://rdp.cme.msu.edu) (Tab. 1). Genus was assigned in all cases except one, in which the length of the partial 16S rRNA allowed identification only to the family level (*Enterobacteriaceae*). Isolates with identical 16S rDNA sequences were further tested using RAPD-PCR and BOX-PCR to confirm or reject clonality. Twenty µL RAPD-PCR analysis consisted of 10 nmol genomic DNA and 25 pmol primer M13

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(Tab. 3) and Finnzymes DyNAzyme II PCR Master Mix (Finnzymes, Espoo, Finland). Reactions were performed using an MJ Research PTC 200 thermocycler (MJ Research, Waltham, Massachusetts) with the following program: 95 °C for 5 min, 45 cycles of 95 °C for 1 min, 36 °C for 1 min and 72 °C for 2 min, 72 °C for 5 min. 25 μL BOX-PCR analysis were performed according to a modified version of the protocol of Urzi (2001). Reactions consisted of 10 nmol genomic DNA, 12.5 pmol primer BOXA1R (Tab. 3), 5% (v/v) DMSO, 2.5 mM $MgCl_2$ and Finnzymes DyNAzyme II PCR Master Mix (Finnzymes). Reactions were performed with the following program: 95 °C for 5 min, 35 cycles of 95 °C for 1 min, 45 °C for 1 min and 72 °C for 2 min, then 72 °C for 10 min. Samples were kept at 4 °C until analysis by agarose gel electrophoresis. Using these molecular typing methods, we reduced the number of unique strains tested from 61 to 37.

Generation of spontaneous rifampicin-resistant mutants

Freshly grown cells were streaked onto an LB agar plate amended with 100 $\mu g.mL^{-1}$ rifampicin (Sigma) (LBR agar) and incubated for 2–3 days. Single rifampicin-resistant colonies were picked, re-streaked for purity, and frozen in LB + 20% (v/v) glycerol at –70 °C.

Genomic DNA isolation

For each environmental strain, single colonies of both the wild-type (WT) and the rifampicin-resistant mutant (Rif^R) strains were inoculated into 5.0 mL LB or LBR, respectively, and incubated for 24 h with shaking. The stationary phase cultures were diluted 1:10 into 9 mL fresh medium and incubated at 28 °C with shaking for approximately 5 h. Cells from 2 mL of each culture were pelleted by centrifugation at 3500× *g* in a tabletop microcentrifuge and frozen at –20 °C. Genomic DNA was extracted from each cell pellet using QIAGEN 20-g genomic tips according to the manufacturer's protocol (Qiagen). DNA was resuspended in 1 X TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) for 18 h at 4 °C. DNA concentrations were measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop, Wilmington, Delware) and final concentrations were adjusted to 100 $ng.\mu L^{-1}$ with TE buffer. DNA samples were stored at –20 °C. Five μL of each DNA preparation was analyzed by agarose gel electrophoresis for general quality of DNA. Both WT and Rif^R DNA were obtained for all 61 strains. Genomic DNA from *A. baylyi* DSM586/*chr*::KTG was isolated using the QIAGEN 500-g genomic tips according to the manufacturer's instructions, and stored at –20 °C. DNA concentration was determined by measuring the absorbance at 260 nm in a SmartSpec^{TM} Plus Spectophotometer (Bio-Rad, Hercules, California).

Filter transformations

Filter transformations were performed using a protocol modified from Ray and Nielsen (2005). Single colonies of wild-type isolates were inoculated into 3 mL LB and incubated at 28 °C for 18 h with shaking. The following day, cultures were diluted 1:10 into 9 mL fresh LB and incubated at 28 °C for an additional 5 h. Cells from 4 mL of this culture were pelleted at 1700× *g* and resuspended in 100 μL sterile 0.85% NaCl. 80 μL of each cell suspension was mixed with 10 μL (1 μg DNA) each of the corresponding wild-type DNA, Rif^R DNA and 0.9% NaCl (saline) in separate 1.5 mL tubes. After briefly vortexing, each mixture was spotted individually onto sterile filters (one filter per treatment) aseptically placed on the surface of an LB agar plate. A fourth filter was kept untreated as a sterility control. In addition, 100 μL of saline was plated onto LB agar to check for sterility. Filters were incubated for 24 h at 28 °C. The bacterial cells were then washed from filters with 3 mL saline in 50 mL polypropylene-tubes and serially 10-fold diluted. Cells of *Pseudomonas stutzeri* JM303 (Carlson et al., 1983) were scraped from filters using a sterile inoculating loop as growth on filters was not perturbed by vortexing alone. Plates were incubated for 2 days at 28 °C prior to manual colony counting. Growth on LBR agar plates for all three treatments for each strain was scored qualitatively to identify any potentially transformable strains. JM303, a derivative of the model transformable bacterium *P. stutzeri* JM300, was included as a positive control in all transformations (Carlson et al., 1983).

In vivo colonization experiments in grass grubs

Acinetobacter baylyi strain BD413 (Juni and Janik, 1969) and *A. baylyi* DSM586/*chr*::KTG (Nielsen et al., 1997a; 1997b) were cultured as described previously (Ray and Nielsen, 2005). *Serratia entomophila* strain 154 was cultured on CTA medium (Starr et al., 1976) at 30 °C. Third instar larvae of New Zealand grass grubs (*Costelytra zealandica* (White)) (Fig. 1a) were collected from pasture in Canterbury, New Zealand, during February 2004, and stored individually in non-sterile-soil-filled wells of 24-well culture dishes without food at 4 °C in the dark. Prior to all experiments, grubs were removed from the soil and checked for signs of disease. Diseased grubs (*i.e.* those with obvious fungal infections or with lightly-colored midgut contents) were excluded from the experiments. To verify health, the grubs were tested for normal feeding behavior. In short, the grubs were individually placed in the wells of 60-well plastic trays and given a

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2 mm × 2 mm × 1 mm carrot piece. Trays were covered with a damp paper towel and placed in a loosely sealed plastic bag at 15 °C in the dark. After 24 h, those grubs which had consumed part or all of their carrot pieces were retained for experiments. Non-feeding grass grubs were assumed to be diseased and were excluded from experiments. Unless otherwise stated, grass grubs were incubated in the dark at 15 °C.

Inocula of competent BD413 cells were prepared from frozen stocks by washing cells with sterile saline to remove glycerol, and resuspending to a concentration of approx. 10^8 competent cells.mL^{-1}. For dissections, 60-well plates containing grass grubs were incubated for up to 1 h on ice prior to dissection to reduce grass grub activity. Dissections were aseptically performed on an ethanol-disinfected wax surface using flame-sterilized equipment. Care was taken to avoid contamination of the alimentary tract with bacteria from the cuticle surface. Alimentary tracts (Fig. 1b) were separated from the head and cuticle by cutting through the musculature at the foregut and anus, respectively. When midgut and hindgut portions were to be examined separately, a cut was made at the pyloric sphincter, which allowed detachment of the two sections without loss of lumen content. Alimentary tracts were immediately transferred to sterile tubes containing 0.5 mL of ice-cold sterile 0.85% NaCl and kept on ice until processing. Alimentary tracts were macerated using sterile microhomogenizing rods, and macerates were sonicated for 7 min and vortexed for 30 s to disrupt bacterial aggregates or attachment of bacteria to tissue or lumen content. Macerates were then centrifuged at 70× *g* in a tabletop microcentrifuge for 1 min to pellet cell debris. For some experiments, alimentary tracts or portions from individual grass grubs were combined into a single sample to reduce variation between individuals.

For recovery of bacteria from the insect gut during colonization experiments, antibiotics were included in LB agar plates, where appropriate, at the following concentrations: rifampicin, 50 μg.mL^{-1}; kanamycin, 50 μg.mL^{-1}; cycloheximide (to inhibit fungal growth) 50 μg.mL^{-1} (Sigma-Aldrich, St. Louis, Missouri). Using a combination of direct oral inoculation of grass grubs with bacteria, two antibiotic selections and one anti-fungal selection, and incubation at 37 °C, we were successful in re-isolating pure cultures of BD413 from the non-sterile gut environment.

Effect of insect gut lumen contents on competence development in *A. baylyi* strain BD413 *in vitro*

Entire alimentary tracts from six grass grubs were collected by dissection, pooled into two groups of three alimentary tracts each, and macerated in 0.5 ml LB broth. To the first macerate sample, 5 μL of a stationary phase culture of BD413 and 5 μL (approx. 3.5 μg) KTG DNA were added. This sample was to test for the ability of BD413 to grow and develop competence in the presence of insect gut contents. To the second macerate sample, only 5 μL BD413 cells were added. This sample served as a negative control for competence development, as no transforming DNA was present. As a positive control, 5 μL BD413 cells and 5 μL KTG DNA were also added to 0.5 mL LB broth without macerates. This was a positive control sample in which BD413 cells were not exposed to insect gut contents during growth and competence development in the presence of KTG transforming DNA. Single samples (n = 1) were incubated at 33 °C overnight with gentle rotation. Single samples (100 μL) from each of the two groups and the positive control were taken at 0, 24 and 48 h after the introduction of BD413 cells, and plated at appropriate dilutions on LBRC agar plates to enumerate total BD413 cells, and LBRC agar plates amended with kanamycin to enumerate transformants. Plates were incubated at 37 °C for 2 days.

Colonization of the insect gut by *A. baylyi* strain BD413

To determine the extent to which *A. baylyi* cells colonize the alimentary tract of grass grub larvae, whole alimentary tracts, both individually and pooled from different larvae sampled at each time point, were analyzed. Non-inoculated grubs were included as negative controls. Inoculum consisting of competent BD413 cells was prepared immediately prior to inoculation of larvae by thawing frozen competent cells, spinning for 10 min at 1130× *g* in a table-top microcentrifuge and discarding the supernatant, and resuspending cells in 300 μL sterile 0.85% NaCl. For the first experiment analyzing **individual** grubs, 20 healthy grass grubs were each injected with approximately 3.6×10^4 CFU of BD413 cells directly into the midgut using a flame-sterilized blunted 20-gauge syringe coupled to a microinjector device. Grubs were incubated at 15 °C in the dark. Three grubs were immediately dissected (day 0) according to the above protocol. After 24 h, all grubs that had died due to midgut puncture during injection were discarded. Remaining grubs were sampled as follows: four grubs on day 1, three grubs on day 2, and four grubs on day 3. Macerates were plated (single replicates) on LBRC agar plates and incubated at 37 °C for 2 days.

For the second experiment analyzing **pooled** samples, 30 healthy grubs were inoculated by microinjection directly into the midgut with approximately 2.1×10^5 competent BD413 cells.larva^{-1} and incubated at 15 °C in the dark. Four grubs were immediately sampled (day 0), and the midgut and hindgut portions were pooled (n = 1) for all four grubs at each sampling and macerated. After 24 h, all grubs that had died due to midgut puncture during injection were discarded. Remaining grubs were sampled

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as follows: four grubs on days 1, 2 and 3, then three grubs on day 4. Pooled fecal pellets were also sampled on day 4. Macerates were plated (single replicates) at appropriate dilutions on LBRC agar plates and incubated at 37 °C for 2 days.

Co-colonization of the insect gut by *Serratia entomophila* strain 154 and *A. baylyi* strain BD413

S. entomophila strain 154 inoculum was prepared by resuspending cells from a CTA plate in sterile 0.85% NaCl. Thirty-four healthy grubs were injected directly into the midgut with 2.5 μL of this suspension (approx. 10^6 cells.larva^{-1}) and incubated overnight at 15 °C. The following day, 2.5 μL of the BD413 inoculum, prepared as described above (4.8×10^5 CFU.larva^{-1}) was injected directly into the midgut of each of the 34 grubs. Four grubs were immediately dissected (day 0) and the midgut and hindgut portions, as well as fecal pellets, for all dissected grubs at each sampling were pooled and macerated together. After normal processing, dilutions in sterile saline were prepared for each macerate, plated on LBRC agar plates, and incubated at 37 °C for 2 days. Remaining grubs were sampled as follows: four grubs on days 1, 2 and 3, and three grubs on day 4.

Determination of genetic competence of *A. baylyi* BD413 cells recovered from insect gut

To determine the genetic competence of the BD413 cells recovered from the larval gut, we performed *in vitro* transformation reactions on BD413-containing grass grub larval gut macerates. Debris-free (*i.e.* spun at 70× *g* for 1 min) macerates were spun at 1130× *g* for 10 min in a tabletop microcentrifuge to pellet all bacterial cells without compromising the membrane integrity of potentially competent BD413 cells. The pellets were resuspended in 1 mL LBR, and 4 μg KTG DNA was added to each sample. Tubes were incubated with gentle rotation for 24 h at 30 °C to facilitate transformation. Cultures were diluted with sterile saline and appropriate dilutions were plated on LBRC agar plates (recipients) and LBRC agar plates amended with kanamycin (transformants) and incubated at 30 °C for 3–5 days.

Stability and transforming activity of DNA recovered from insect lumen

Approximately 3.85 μg KTG DNA (~0.77 μg.μL^{-1}) was introduced by microinjection directly into either the foregut or the midgut of healthy grass grub larvae. One each, of foregut-injected and midgut-injected larvae, were sampled at 2, 4, 24, 47 and 71 h after injection. One un-inoculated control larva was sampled only at the first sampling point (2 h). Fecal pellets from DNA-injected larvae were collected at 47 h post-inoculation. At sampling time 71 h, we observed that two of the grubs appeared to be diseased, as shown by their light-colored lumen contents. The two diseased grubs (one foregut-injected and one midgut-injected) were sampled along with two healthy foregut- and midgut-injected grubs at this sampling point. After dissection, midgut and hindgut sections were homogenized in ice-cold sterile saline, sonicated for 7 min, vortexed for 30 s and centrifuged for 1 min at 70× *g*, total DNA was isolated from each sample using FastDNA SPIN Kit (for soil) (QBiogene, Morgan Irvine, California) according to the manufacturer's instructions. Total DNA concentration was determined by measuring absorbance at 260 nm, and samples were adjusted to 10 ng.μL^{-1} with 10 mM Tris-Cl buffer, pH 8.0. One μL of each DNA sample was spotted onto the surface of an LB agar plate to confirm sterility. Samples were stored at –20 °C until PCR analysis. One hundred ng total DNA was analyzed by PCR for the presence of the KTG cassette using primer sets IB3/4 and NptI-IConner (Tab. 3). Reaction conditions were as follows: 12.5 μL AmpliTaq Gold Master Mix, 5 pmol each primer, in 25 μL total reaction volume. PCR was performed on an Eppendorf thermocycler with the following parameters: denaturing at 94 °C for 5 min, 30 cycles of 94 °C for 30 s/55 °C for 30 s/72 °C for 90 s, followed by a final elongation at 72 °C for 7 min and storage at 4 °C until analysis. Ten μL of each PCR product was analyzed by agarose-gel electrophoresis and by ethidium bromide staining. One μL of each DNA preparation was spotted onto an LB agar plate and incubated for 48 h at room temperature to ensure sterility.

To test whether the DNA recovered from the grass grub alimentary tract retained transforming activity, 10^8 competent BD413 cells were incubated with 10 μL (approx. 1 μg) of each DNA preparation (single replicate). After incubation at 33 °C with gentle rotation for 21 h, cells were recovered by centrifugation at 1700× *g* for 5 min in a tabletop microcentrifuge and resuspended in 350 μL LB. One hundred μL of undiluted cell suspension was plated in duplicate on LBR agar plates amended with kanamycin to enumerate kanamycin-resistant transformants. Undiluted suspension (100 μL) was serially 10-fold diluted in sterile saline to 10^{-6} and plated on LBR agar plates to enumerate recipients. Plates were incubated at 37 °C.

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