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Enhancement of bacterial competitive fitness by apramycin resistance plasmids from non-pathogenic *Escherichia coli*

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The study of antibiotic resistance has in the past focused on organisms that are pathogenic to humans or animals. However, the development of resistance in commensal organisms is of concern because of possible transfer of resistance genes to zoonotic pathogens. Conjugative plasmids are genetic elements capable of such transfer and are traditionally thought to engender a fitness burden on host bacteria. In this study, conjugative apramycin resistance plasmids isolated from newborn calves were characterized. Calves were raised on a farm that had not used apramycin or related aminoglycoside antibiotics for at least 20 months prior to sampling. Of three apramycin resistance plasmids, one was capable of transfer at very high rates and two were found to confer fitness advantages on new *Escherichia coli* hosts. This is the first identification of natural plasmids isolated from commensal organisms that are able to confer a fitness advantage on a new host. This work indicates that reservoirs of antibiotic resistance genes in commensal organisms might not decrease if antibiotic usage is halted.

Keywords: conjugative plasmid; antibiotic resistance; fitness advantage; commensal

1. INTRODUCTION

Research into antibiotic resistance has previously been focused on organisms that are pathogenic to humans or animals. However, the development and persistence of antibiotic resistance in commensal organisms is of concern because they are thought to act as a reservoir of resistance genes (Schwarz *et al.* 2001). Resistance genes are commonly carried on conjugative plasmids, which are circular molecules of extra-chromosomal DNA capable of autonomous replication and horizontal transfer. Genes conferring antibiotic resistance can spread between commensal and pathogenic organisms by conjugation (Mizan *et al.* 2002). Some conjugative plasmids conferring antibiotic resistance have broad host ranges and have been documented to be capable of transfer between different species and even genera (Amabile-Cuevas & Chicurel 1992).

The carriage of conjugative resistance plasmids is generally thought to engender a competitive fitness disadvantage on host bacteria in the absence of selective pressure for resistance phenotypes (Goodwin & Slater 1979). Therefore, a reduction in antibiotic use could result in a decrease in the prevalence of corresponding resistance determinants. Yet resistance plasmids that confer a fitness advantage on their host bacterium have been reported (Lenski *et al.* 1994; Enne *et al.* 2004; Dionisio *et al.* 2005). The detection of antibiotic resistance plasmids capable of enhancing bacterial fitness is of concern.

Previous studies have focused on plasmids from clinical pathogens, common laboratory plasmids or cloning vectors. There appear to be no studies to date that have determined fitness costs associated with plasmids carried by commensal organisms, despite the fact that commensals are thought to be important in maintaining resistance reservoirs.

The current paper assessed whether conjugative plasmids conferring apramycin resistance (apr^R) isolated from commensal *Escherichia coli* isolates, increased or decreased the competitive fitness of new host cells.

2. MATERIAL AND METHODS

(a) *Strains, plasmids and conjugations*

Apr^R plasmids (pUK2001, pUK2002 and pUK2003) were obtained from commensal *E. coli* of newborn calves (never treated with apramycin or related aminoglycosides), and tetracycline and apramycin resistance genes identified using the methods of Aminov *et al.* (2002) and Yates *et al.* (2004) respectively. Plasmids were transferred by conjugation into *E. coli* K12 strains MG1655 and J62-2 by standard procedures (Provence & Curtiss 1994). Transfer rates were measured in triplicate between J62-2 and *E. coli* J53 (Bachmann 1972), by the method of Simonsen *et al.* (1990).

A kanamycin-resistant MG1655 strain (designated MG1655kan) was constructed using a temperature sensitive allelic exchange plasmid to replace the *lacZY* genes with a kanamycin (kan) resistance cassette. Briefly, primers (Roe *et al.* 2003) were used to amplify strain-specific *lac* sequences from MG1655 that were cloned into plasmid pIB307 (Blomfield *et al.* 1991). A kan resistance cassette was cloned in between the two *lac* specific sequences and allelic exchange performed using standard methods (Hamilton *et al.* 1989). Successful replacement of the *lacZY* genes with the kan cassette was verified using LR–PCR using primers previously described (Roe *et al.* 2003).

(b) *Fitness measurements*

Pairwise competition experiments of plasmid-bearing MG1655 against the plasmid-free isogenic competitor, MG1655kan, were performed in Davis Mingioli broth (DM) supplemented with glucose (0.4%), to limit conjugation (electronic supplementary material), by a modification of Lenski *et al.* (1994). The use of MG1655 strains permitted the quantification of both competing strains on the same agar detection plate, reducing the potential for error introduced by pipetting. Mono-cultures of competing strains were preconditioned in DM overnight prior to the experiment start. At time zero, cultures were mixed at a volumetric ratio of 1 : 1 and this mixture diluted 1 : 100 into fresh DM broth. After incubation for 24 h at 37 °C with gentle shaking (150 r.p.m.), cultures were diluted 1 : 100 into fresh media. This procedure was repeated daily for 5 days. Densities of plasmid-harbouring MG1655, plasmid-free MG1655kan and transconjugant cells were estimated at time zero and every 24 h by serial dilution in saline and selective plating. S-gal plates (sigma) were used for estimates of plasmid-carrying (black) and plasmid-free cells (white), and MacConkey plates supplemented with 40 mg l^{-1} apramycin and 32 mg l^{-1} kanamycin, for quantification of any transconjugants. Six replicate competition assays were performed for each plasmid. The selection coefficient (S) was estimated as described by Lenski *et al.* (1991) and was calculated as the growth of plasmid-carrying MG1655 relative to MG1655kan; a positive relative fitness demonstrates a growth advantage of plasmid-carrying MG1655 compared to MG1655kan.

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The selection rate constant, r (d^{-1}) was calculated by regressing the natural log-transformed ratio of plasmid-bearing to plasmid-free cell densities against time (Lenski *et al.* 1994), over the 5 day period.

(c) *Statistical analysis*

Effects of plasmid, host, media (LB or DM) and detection plate (MacConkeys or MacConkeys supplemented with 16 mg l^{-1} apramycin) on numbers of cfu ml^{-1} during 5 day competition assays were analysed with linear mixed effects models using S-Plus (Insightful, Seattle). Data for which residuals were not normally distributed, were log-transformed before the analysis was performed. Different replicate flasks were entered as random effects to account for both variation between replicate experiments and lack of independence between samples. Type of detection plate was included in the model as a fixed effect to determine whether plasmid segregation was occurring.

3. RESULTS

All three apr^R plasmids carried the apramycin resistance gene, *aac(3)IV*, but transferred at different rates (table 1). pUK2002 and pUK2003 also carried the tetracycline resistance determinant, *tetB*.

Plasmids pUK2001 and pUK2002 conferred relative fitness advantages on MG1655 that were significantly greater than the advantage of this strain over its kanamycin resistant counterpart ($t_6 = -5.56$, $p < 0.001$; $t_6 = -7.27$, $p < 0.001$, respectively; table 2). In contrast, pUK2003 conferred a relative fitness cost on MG1655 ($t_7 = 7.55$, $p < 0.001$). The control for these assays was the direct competition of MG1655 and MG1655kan. A mean selection coefficient of $S = 0.043 \pm 0.024$ revealed that insertion of the kanamycin resistance gene into MG1655 marginally reduced the competitive fitness of the strain (table 2). If there is no difference in the growth rate of two competing cultures but one has a higher inoculum, the difference in competing cell densities may be amplified with each repeat passage. To ensure that variation in the number of bacteria present in the initial cultures did not influence the outcome of the competition, control experiments were repeated with an initial ratio of 1 : 2 and of 2 : 1 MG1655 to MG1655kan. The selection rate constants of six replicate competitions did not differ significantly when initial ratios of 1 : 2 or 2 : 1 were used ($t_6 = -0.386$, $p = 0.712$).

Selection rate constants for MG1655 carrying pUK2001 and pUK2002 were significantly different from the control ($t_{10} = -4.87$, $p < 0.001$; $t_{10} = -5.96$, $p < 0.001$, respectively). Plasmid segregation of pUK2001 or pUK2002 was discounted, as changes in cfu ml^{-1} with time did not differ significantly when measured on S-gal plates or MacConkey plates supplemented with 16 mg l^{-1} apramycin ($t_{45} = 0.96$, $p = 0.342$; $t_{112} = 1.18$, $p = 0.24$, respectively).

4. DISCUSSION

Resistance plasmids are threatening the successful management of both human and animal bacterial infections. Maintenance of resistance plasmids in commensal organisms is of concern because commensals can act as a reservoir of resistance genes that can be transferred to more pathogenic organisms (Schwarz *et al.* 2001). Despite this fact, the cost or advantage associated with the carriage of resistance plasmids has not previously been studied in wild-type plasmids from commensal organisms.

Table 1. Mean transfer rates and resistance gene carriage of the apr^R plasmids studied.

plasmid	size (kb)	resistance genes present	transfer rate (log (ml per cell h^{-1}))
pUK2001	91	*aac(3)IV*	-8.391 ± 0.726
pUK2002	115	*aac(3)IV*, *tetB*	-10.400 ± 0.243
PUK2003	181	*aac(3)IV*, *tetB*	-14.779 ± 0.550

Table 2. Selection coefficients and selection rate constants of MG1655 harbouring apr^R plasmids, in direct competition with MG1655kan. (Mean values of r and S from six assays are given per competition $\pm$ twice the standard error.)

competing strain (against MG 1655kan)	selection rate (constant) (r) (d^{-1})	selection coefficient (S) after 5 days
MG1655 pUK2001	0.223 ± 0.071	0.351 ± 0.108
MG1655 pUK2002	0.334 ± 0.096	0.412 ± 0.099
MG1655 pUK2003	-0.069 ± 0.038	-0.057 ± 0.011
MG1655 (control)	0.039 ± 0.029	0.043 ± 0.024

In this work, apramycin resistance plasmids were isolated from newborn calves reared on a farm where apramycin (or related aminoglycosides) had not been used for at least 20 months (when treatment records commenced). One of the conjugative plasmids detected had a relatively high transfer rate of 4.06×10^{-9} ml cell^{-1} h^{-1} (log -8.391). Gordon (1992) reported an average transfer rate of plasmid R1 between wild-type *E. coli* strains of 1.3×10^{-15} ml per cell h^{-1} (Gordon 1992). Although transfer of R1, between *E. coli* K12 strains (as used in the current work) has been found to be at least 1000 fold faster than between wild-type *E. coli* strains (Gordon 1992), the transfer rate of pUK2001 is still comparatively high.

The plasmids studied in this work are natural plasmids that were isolated from wild-type commensal *E. coli* and so are likely to have undergone evolution in these commensal hosts and perhaps other gut bacteria present in the calves. Dahlberg & Chao (2003) previously demonstrated that the well-characterized plasmids R1 and RP4 initially decreased the fitness of an *E. coli* K12 strain, but through *in vitro* evolution of the plasmid-containing bacteria without antibiotic selection, this cost could be ameliorated. Similarly, Dionisio *et al.* (2005) describe how the *in vitro* evolution of R1 in an *E. coli* K12 strain resulted in an evolved plasmid able to dramatically increase fitness of other cells such as *Salmonella*. The fitness advantages of pUK2001 and pUK2002 on *E. coli* K12 MG1655 indicates that *in vivo* evolution of wild-type plasmids can result in an ability to confer fitness advantages on new hosts.

During 5 days of pairwise competition, the average selection rate constant of pUK2002 was found to be $r = 0.334 \pm 0.096$ d^{-1}. Similar selection rate constants ($r = 0.216 \pm 0.043$ d^{-1}) have been described by Lenski *et al.* (1994) for a 2.9 kb plasmid conferring

tetracycline resistance. Lenski *et al*. (1994) suggest that the tetracycline resistance gene (*tetC*) was the factor responsible for the enhanced fitness with plasmid carriage in coevolved hosts. Although plasmid pUK2002 carries a tetracycline resistance determinant (*tetB*), plasmid pUK2003 also has this gene but does not confer a fitness advantage. Therefore, in contrast to the results of Lenski *et al*. (1994) it is unlikely that this tetracycline resistance gene plays a significant role in the conferred fitness of these two plasmids.

In summary, this is the first identification of natural plasmids isolated from commensal organisms able to confer a fitness advantage on a new host. The fact that some natural antibiotic resistance plasmids enhance host fitness is noteworthy and indicates that reservoirs of antibiotic resistance genes in commensal organisms might not decrease if antibiotic usage is halted.

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