Section 2.1 : Non-Technical (Layperson's) Executive Summary

Project Title: Survival of ingested DNA in the gut and the potential for genetic transformation of resident bacteria

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There is increasing interest in Genetically-Modified (GM) plant material and microorganisms as means to improve both food quality and the efficiency of food production. This project was designed to address scientific and public concerns over the fate of GM DNA in the human gut following ingestion. To this end we investigated the survival of ingested DNA *in vitro* under simulated gut conditions and *in vivo* in the rat gut, and also the potential for genetic transformation of resident oral and gut bacteria by surviving DNA. This research would therefore provide the public and the Food Standards Agency (FSA) with important information regarding the survival of GM plant DNA and GM micro-organism survival under gut conditions, and may be used to inform the safety assessment of GM foods carried out on behalf of the FSA in the UK by the Advisory Committee on Novel Foods and Processes (ACNFP).

The survival of DNA in the human mouth was determined *in vivo* by a human volunteer, after receiving the necessary ethical committee approval. The half-life of DNA *in vivo* was only 6 seconds and the concentration had decreased ~ 100-fold after only 60 seconds exposure. DNA was much less stable under *in vivo* conditions than was apparent from previous *in vitro* experiments with human saliva. Nevertheless, when tested *in vitro*, sufficient partially degraded DNA survived to bring about genetic transformation, (uptake of free DNA and its heritable incorporation into the bacterial genome) to antibiotic resistance, of a strain of the oral bacterium *Streptococcus gordonii.*,Since *S. gordonii* is capable of transformation in human saliva without any special pre-treatment, the possibility must exist for transformation of related bacteria to occur *in vivo*. In the course of this work we established that a lower gut strain of another Streptococcus species, *S. bovis*, which has been implicated in disease causation, was similarly capable of natural transformation. Transformation of both *S. gordonii* and *S. bovis in vitro* was observed in saliva, but not under simulated small intestinal or colonic conditions. Selected strains of *Excherichia coli* (ubiquitous throughout the gut) and *Helicobater pylori* (gastric bacteria) can be transformed *in vitro*. We were unable to transform gut isolates of these bacteria under conditions simulating those of the mouth or stomach. DNA survival will of course vary with different gut conditions. *In vitro* data obtained here identified some food components that may enhance DNA survival.

In most cases, fragments of GM DNA in food will not be capable of propagating themselves in a gut or oral bacterium even if they are taken in by bacterial cells. They are only likely to become heritably acquired if they insert into the host chromosome. This could occur, if the GM DNA and bacterial chromosome share regions with identical sequences. The transformation of two native gut bacteria, *S. gordonii* and *S. bovis*, was therefore investigated using DNA that is able to integrate into the bacterial chromosome as a result of matching sequences provided by an antibiotic resistance marker gene. Higher transformation efficiencies were obtained *in vitro* with DNA that was able to integrate into the bacterial chromosome than with self-replicating plasmid DNA that had no sequence match with the bacterial chromosome. We did not detect transformation *in vitro* using linear DNA that possessed only a single region of matching sequence, which is, arguably, the most likely state for GM DNA in food. We did, however, detect transformation for genes that were flanked on both sides by sequences that match the bacterial chromosome.

When naked GM DNA was fed to rats, DNA from marker genes remained detectable in the rat faeces for up to 79 hours. Preliminary data indicated the possibility that transformation of gut bacteria by the added GM DNA might occur in the rat gut, but rigorous confirmation is lacking. In order to study the fate of GM bacteria under gut conditions, marked strains were added to fermentor simulations of the human colon, or fed to rats. GM *Lactococcus lactis* were rapidly eliminated from the fermentor, suggesting active killing by the resident microflora, but for a tail population that was able to establish itself in the fermentor at low numbers. This tail population was attributed to adaptation of the introduced strain rather than to gene transfer events. GM *Enterococcus faecalis* were fed to laboratory rats and were able to survive in the gut for 11-13 days. During this time no evidence of transfer of the GM marker genes to the native microflora of the rat gut was observed.

In conclusion, there is a possibility of rare acquisition of GM sequences by resident bacteria in the mouth or gut. The probability of such events is influenced by the design of GM constructs since it depends largely on the presence of matching sequences in the host bacterium and GM DNA. This conclusion must however be put firmly into its evolutionary context. Humans have consumed huge amounts of DNA in food throughout evolutionary history, and the possibility of gene acquisition by resident oral and gut bacteria has always existed.

Technical Report

FSA Project Code: FSG01007

DNA Survival in the Mouth and the Potential for Transformation of Competent Oral Bacteria

The plasmid pVACMC1 carries a convenient selectable erythromycin resistance marker gene and a cloned cellulase gene, and can replicate in Gram positive bacterial hosts (14). We measured degradation of the plasmid pVACMC1 *in vivo* in the mouth of one human volunteer (after obtaining the relevant ethical committee approval) as shown in Figure 1. To achieve this 10 μg DNA was taken into the mouth and saliva sampled after 0, 10, 30, and 60 seconds. DNA degradation was measured by competitive PCR, using a 510 bp target sequence, as described previously (14). Degradation was very rapid *in vivo*, with a 2 log decrease in the concentration of remaining DNA after only 60 seconds exposure. This is approximately 4 times more rapid than estimated previously under *in vitro* conditions (14;15). Nevertheless, this degraded plasmid DNA was still able to transform competent cells of *S. gordonii* DL1 *in vitro* (Figure 1). There was a good correlation between the rate of DNA degradation and the decrease in transformation frequency (Figure 1) (15).

Mouth washings (after pVACMC1 introduction) were plated on selective medium (BHI agar + 10 μg/ml Em + 0.1% (w/v) carboxymethylcellulose) to determine whether any transformation of the resident oral microflora had occurred in the mouth. No erythromycin resistant, endoglucanase-producing bacteria were detected. However, it is anticipated that such gene transfer events would be very rare and therefore very difficult to detect using this method, owing to the high background incidence of erythromycin resistant bacteria in the oral cavity.

Effect of Food Components on the Degradation of DNA in Human Saliva

The effect of food and food components on pVACMC1 plasmid DNA degradation *in vitro* was also studied using the competitive PCR approach of Mercer *et al* (14). Initial experiments were carried out with different dairy products (Table 1). Degradation of DNA was not reduced by the addition of skimmed, semi-skimmed, or full fat milk to saliva, especially when compared to an average of 52% degradation in

Figure 1 - Fate of Free pVACMC1 DNA in the Human Mouth

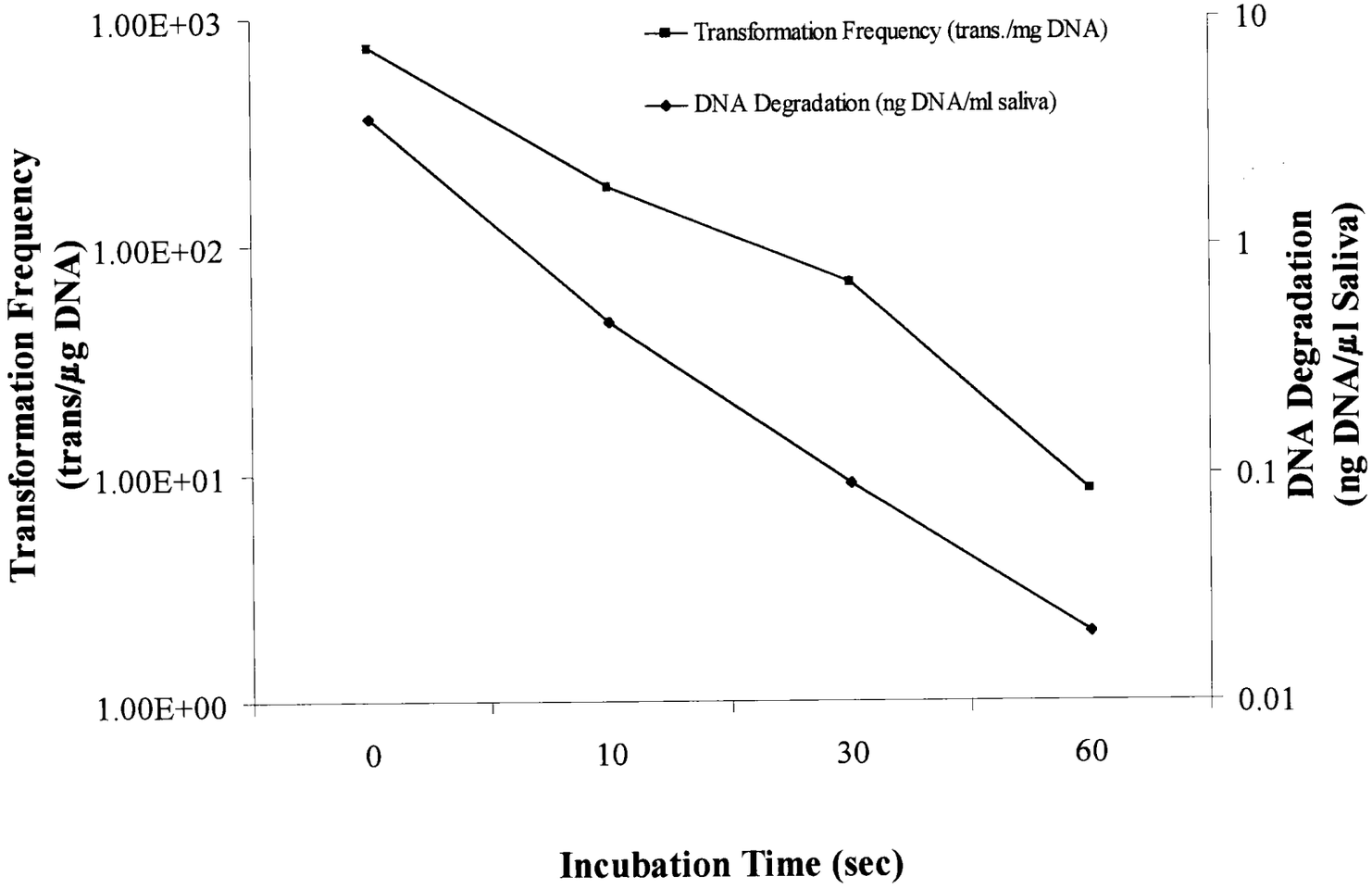


Table 1. Effect of saliva and food supplements on degradation of pVACMC1 plasmid DNA

	DNA Concentration (ng/μl)[1]		DNA Degradation
Food Type	**t = 0 sec**	**t = 10 sec**	**(% of t = 0 sample remaining after 10 sec)**
Saliva alone	**6.3**	**3.3**	**52**
Skimmed Milk	**4.6**	**1.3**	**71**
Semi-skimmed Milk	**0.9**	**0.2**	**74**
Full Fat Milk	**3.5**	**1.5**	**57**
Powdered Milk	**2.5**	**3.8**	**152**
Low Fat Yoghurt	**NE[2]**	**0.5**	**-**
Biological Yoghurt	**2.5**	**NE[2]**	**-**
Egg	**NE[2]**	**NE[2]**	**-**
Olive Oil	**NE[2]**	**NE[2]**	**-**

[1] All results are means of triplicate competitive PCR's

[2] NE = No extraction of DNA possible

saliva alone from the same volunteer after 10 seconds. Binding of DNA to food components may explain the differences in apparent initial concentrations of added DNA, especially for semi-skimmed milk. Competitive PCR was attempted with DNA that had been exposed to saliva and yoghurts, egg and olive oil but extraction of amplifiable DNA was not possible. This may be because selective binding of DNA makes it unavailable for PCR amplification.

Transformation and chromosomal integration of GM DNA via a region of homology provided by a common antibiotic resistance gene

Most of this work is described in the manuscript "Transformation of an oral bacterium via chromosomal integration of free DNA in the presence of human saliva" (15). Most plasmids used in genetic modification have limited bacterial host ranges that minimise the bacteria in which they might be able to replicate in nature. The host range for acquisition is potentially far greater, however, if any homology exists between the plasmid and the bacterial chromosome, that can lead to integration into the chromosome (11). Therefore, we wanted to study events that may lead to the integration of GM DNA into the chromosome of *S. gordonii* DL1. In order to study chromosomal integration events *in vitro*, we decided to use a red shifted mutant of the green fluorescent protein (*gfp*) gene as a model transgene since this allows convenient and unambiguous identification of transformants by fluorescence and by PCR with specific primers (18). A 458bp fragment of the tetracycline resistance gene *tet*(M) was cloned into a plasmid carrying *gfp* that is able to replicate in *E. coli* but not in Gram-positive hosts to form the construct pKPSPgfp-int (19). The complete *tet*(M) gene was also introduced into the chromosome of *S. gordonii* DL1 on the conjugative transposon Tn*916* using the plasmid pAM120 to form the strain *S. gordonii* DL1/Tn*916* (15).

Naturally competent *S. gordonii* DL1/Tn*916* cells were transformed by pKPSPgfp-int *in vitro* at high efficiencies (1.2×10^{-2} per recipient). This is assumed to occur by the mechanism proposed in Figure 2. This contrasts with an efficiency of transformation of 2.9×10^{-4} per recipient for the related plasmid pVACMC1, using the same batch of competent cells, (Table 2). pVACMC1 is capable of replication in *S. gordonii* DL1 but lacks homology with the chromosome, and therefore cannot undergo chromosomal integration. Apparent transformation efficiencies may have been increased by the fact that the incoming plasmid pKPSPgfp-int is integrated into

Figure 2 - Proposed Mechanism of Chromosomal Integration using Plasmid DNA

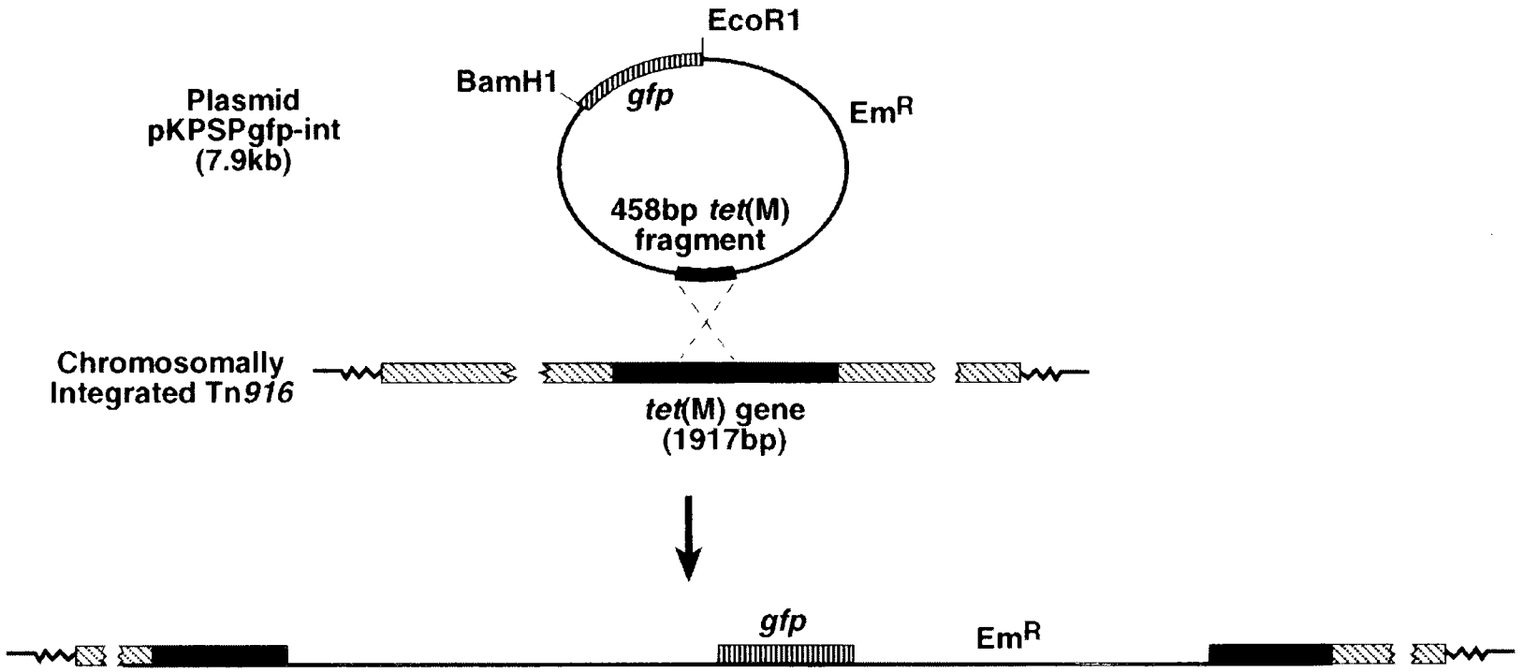


Table 2 - Comparison of Transformation Efficiencies for Different DNA Types

Transforming DNA	Transformation Efficiency (tr./parent cell)
pVACMC1	2.9×10^{-4}
pKPSPgfp-int	1.2×10^{-2}
E. faecalis/Tn*916/gfp*	5.1×10^{-5}
Chromosomal DNA	

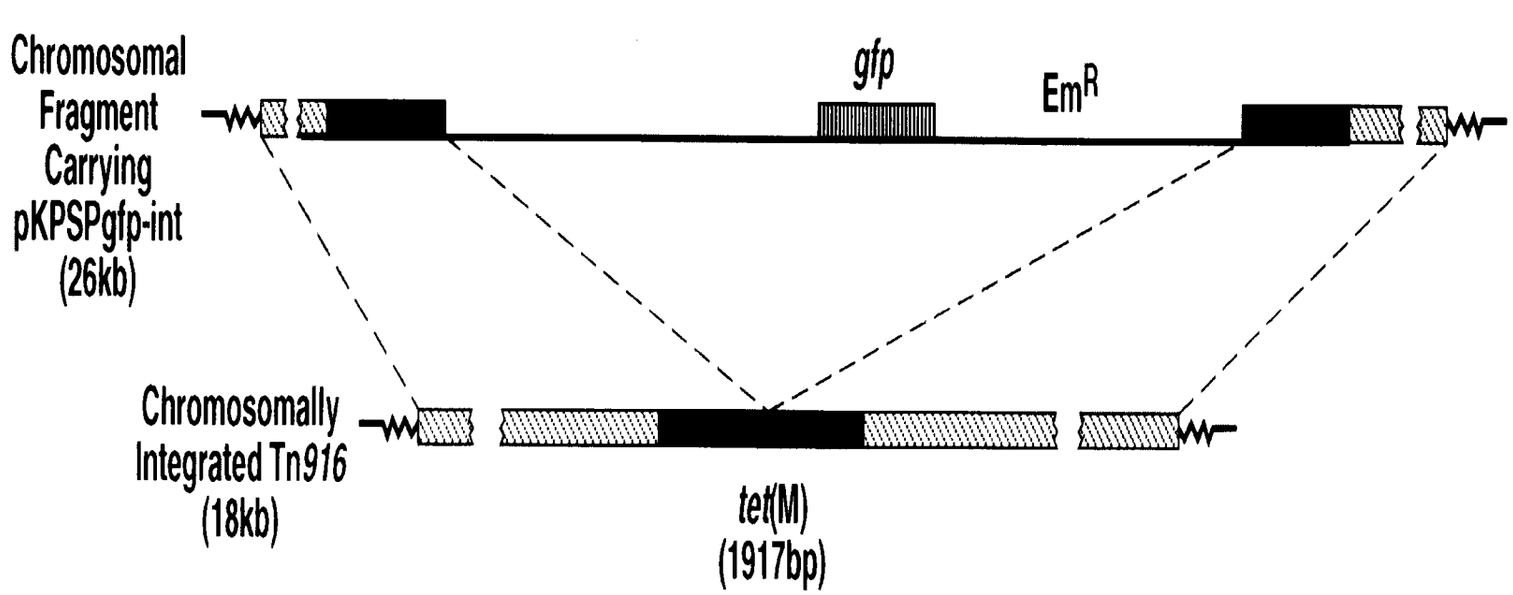
the transposable element Tn*916* and may therefore spread further by conjugation. However, this must be considered realistic as antibiotic resistance genes are commonly found on such mobile elements in nature.

Integration of linear DNA fragments

Genetically modified DNA is unlikely to be approved for use when carried on a plasmid because of the presumed increased risk of transfer of the plasmid/GM gene to an alternative host. Therefore, any free DNA in the diet in the future is more likely to be chromosomal than plasmid in origin. A third transformation experiment, using the same batch of competent cells, examined the situation in which regions of DNA homology are present on both sides of the *gfp* marker. Chromosomal DNA from an *E. faecalis* strain that carries pKPSPgfp-int integrated into Tn*916* was able to transform *S. gordonii* DL1/Tn*916*. In this case the assumed mechanism is shown in Figure 3. The transformation efficiency (5.1×10^{-5} transformants per parent cell) was only 1,000-fold lower than with plasmid pKPSPgfp-int (Table 2), despite the fact that the number of *gfp* gene copies in one gram of pKPSPgfp-int plasmid DNA is predicted to be some 200,000 times greater than for chromosomal DNA. This high rate of transformation may be due in part to the length of Tn*916* (18,032 bp) which thus provides a very extensive region of homology for recombination events. In addition it is again possible that conjugal transfer of Tn*916-gfp* from transformed to non-transformed cells may have boosted the apparent transformation frequency. The transformation efficiency observed with chromosomal DNA is only 10-fold lower than the plasmid transformation efficiency observed with pVACMC1, despite the far lower copy number of transforming DNA within the chromosomal DNA. Conjugal transfer of the Tn*916/gfp* construct was shown from *S. gordonii* DL1/Tn*916/gfp* to *L. lactis* IL1403. No transformants were detected when *S. gordonii* DL1, which lacks the Tn*916* sequence, was used as recipient, confirming that the initial transformants must have arisen by homologous recombination. Transformation of *S. gordonii* DL1/Tn*916* was also observed with chromosomal DNA prepared from *Lactococcus lactis* IL1403 that carries pKPSPgfp-int integrated into Tn*916* (4.7×10^{3} transformants per μg DNA).

Linearisation of chromosomal DNA from *E. faecalis* or *L. lactis* carrying pKPSPgfp-int integrated into Tn*916* with *Not*I produced chromosomal DNA

Figure 3 - Proposed Mechanism of Chromosomal Integration using Chromosomal Transforming DNA



fragments with a region of homology on one side of the *gfp* transgene only, and this DNA failed to generate detectable transformants (Table 3). Linearisation of pKPSPSgfp-int with *Eco*RI, *Bam*HI or *Eco*RV also resulted in no transformants being detected (Table 3).

Nature of the plasmid integration event

The extent of pKPSPgfp-int integration into the chromosome of *S. gordonii* DL1/Tn*916* was determined by PCR using combinations of the *tet*(M) and *gfp* primers (Figure 4). The 3.9 and 4.7 kb PCR products obtained are consistent with integration of the whole of pKPSPgfp-int into the chromosome. However, the sensitivity limitations of agarose gel electrophoresis do not allow us to tell exactly how much DNA has been integrated into the chromosome as this method is not sufficiently sensitive to detect the loss of 30 or fewer bases during DNA uptake or processing. Sequencing across the integration junction, however, showed that no terminal DNA had been lost during DNA uptake and homologous recombination into the chromosome.

Transforming DNA enters streptococcal cells in a single-stranded linear form and it has been hypothesised that transforming plasmid DNA must re-circularise and become double-stranded before it integrates into the chromosome. It appears likely therefore that pKPSPgfp-int re-circularises prior to integration. Consistent with this, pKPSPgfp-int linearised with *Eco*RI, *Eco*RV or *Bam*HI failed to yield detectable erythromycin resistant transformants since integration of the linear fragment by a single crossover would normally result in chromosome breakage. Integration of genes flanking *tet*(M) in linear DNA fragments, therefore, appears unlikely unless there is a region of homology with the chromosome on both sides of the *gfp* gene.

Requirements for chromosomal integration of plasmid DNA

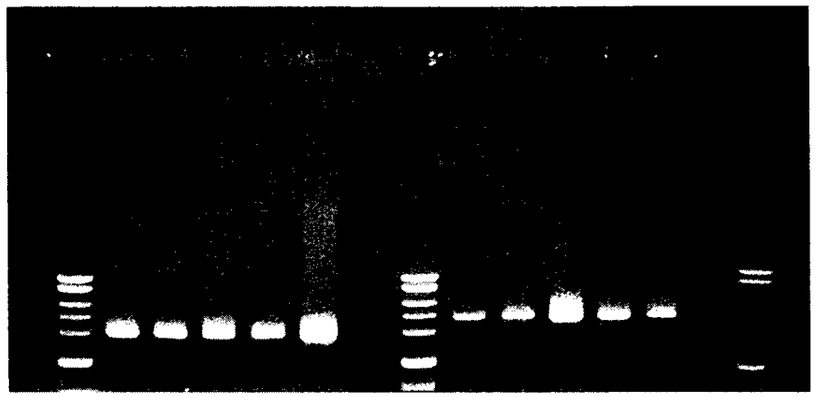
The work so far has demonstrated that a 458 bp region of homology is sufficient to allow the integration of 7.5 kb (7500 bp) of non-homologous DNA. Thus, a relatively small fragment of homologous DNA can promote the integration of a piece of heterologous, e.g. genetically modified DNA, at least 16 times greater in size. We were interested in what degree of homology would allow integration of DNA into the chromosome of *S. gordonii* DL1/Tn*916*.

Table 3 - Effect of Restriction Digestion on Transformation

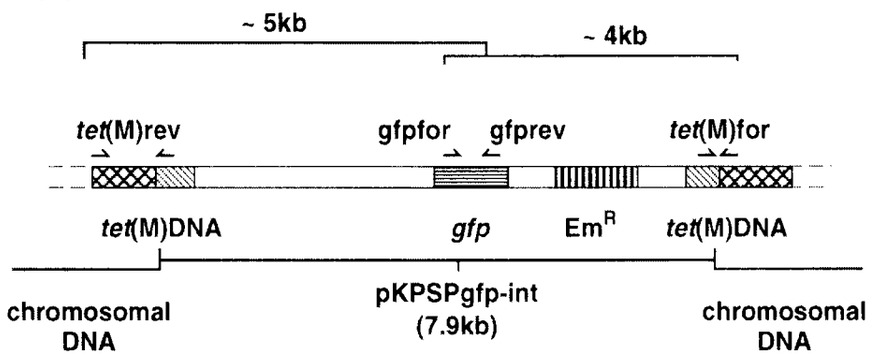
Transforming DNA	Transformation Efficiency (tr./parent cell)
pKPSPgfp-int	2×10^{-4}
pKPSPgfp-int/*Eco*RI (or *Eco*RV or *Bam*H1)	0
L. lactis/Tn*916*/*gfp* chromosomal DNA	4×10^{-5}
L. lactis/Tn*916*/*gfp* chromosomal DNA/*Not*I	0

Figure 4 - PCR Analysis of the Integration of pKPSPgfp-int into the Chromosome of *S. gordonii* DL1/Tn*916*

(a)



(b)



The 458 bp *tet*(M) fragment of pKPSPgfp-int was replaced with identically sized fragments of the related tetracycline resistance genes *tet*(O) and *tet*(W) to form the plasmids pKPSPgfp-int(O) and pKPSPgfp-int(W), respectively. The *tet*(O) and *tet*(W) fragments show 76.9% and 69.7% DNA sequence identity over the 458 bp region of homology (Figure 5). Transformation was attempted with the 3 plasmids pKPSPgfp-int, pKPSPgfp-int(O) and pKPSPgfp-int(W) on the same batch of *S. gordonii* DL1/Tn*916* competent cells. No transformants were detected using the pKPSPgfp-int(O) and pKPSPgfp-int(W) plasmids, whereas a transformation efficiency of 6.7×10^{-3} was obtained with pKPSPgfp-int. This therefore indicates that the region of homology required for chromosomal transformation must show >76.9% overall DNA sequence identity (within a 458 bp region of homology) to allow transformation to occur. Reports in the literature (12) indicate that a region of homology of only 27 bp is sufficient to allow chromosomal transformation. However, in the case of pKPSPgfp-int(O) and pKPSPgfp-int(W) the longest unbroken DNA sequence identical to that of *tet*(M) in the recipient is only 13 bp. This is probably why transformation was not detectable even though a much longer fragment of DNA was employed. It is reported that prevention of recombination in streptococci is prevented by the DNA mismatch repair system, but this system can be overrun if DNA concentrations are very high. Even when DNA concentrations of pKPSPgfp-int(O) and pKPSPgfp-int(W) as high as 50 μg/ml were used no transformation was observed. When the same experiment was carried out with pKPSPgfp-int transformation efficiencies did not increase significantly above 1.2×10^{-2} ($p<0.5$) when the DNA concentration was >15 μg/ml.

Competence induction and transformation of *S. gordonii* DL1 in simulated gut contents

Transformation of *S. gordonii* DL1 and *S. gordonii* DL1/Tn*916* has already been demonstrated in human saliva (14;15). Transformation was therefore attempted in gut contents simulating other regions of the gastro-intestinal tract. These included simulated stomach contents (saliva acidified with 5M HCl), over a pH range of 1-7, simulated small intestinal contents (pancreas acetone solution) and simulated colonic contents (10% (w/v) human faecal slurry) (Figure 6). Transformation with the same batch of competent cells was also carried out in the presence of saliva and sterile-

Figure 5 - Sequence Homology Between *tet*(M), *tet*(O) [76.9% homology] and *tet*(W) [69.7% homology]

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tetM    GCTCATGTTGATGCGGGAAAAACTACCTTAACAGAAAGCTTATTATATAACAGTGGAGCG  60
tetO    GCTCACGTTGACGCAGGAAAGACAACATTAACGGAGAGTTTATTGTATACCAGTGGTGCA  60
tetW    GCCCATGTAGACGCTGGAAAGACGACCTTGACGGAGAGCCTGCTATATGCCAGCGGAGCC  60
        ** ** ** ** ** ***** ** ** ** ** ** **  *  * ***  *** ** **

tetM    ATTACAGAATTAGGAAGCGTGGACAGAGGTACAACGAAAACGGATAATACGCTTTTAGAA  120
tetO    ATTGCAGAACCAGGGAGCGTAGATAAAGGCACAACAAGGACAGATACAATGAATTTGGAG  120
tetW    ATTTCAGAACCGGGGAGCGTCGAAAAAGGGACAACGAGGACGGACACCATGTTTTTGGAG  120
        *** *****   ** ***** ** * *** ***** *  ** ** *  * *  *** **

tetM    CGTCAGAGAGGAATTACAATTCAGACGGCGATAACCTCTTTTCAGTGGAA-AAATACTAA  179
tetO    CGTCAAAGGGGAATCACTATCCAGACAGCAGTGACATCTTTTCAGTGGGA-GGATGTAAA  179
tetW    CGGCAGCGTGGGATTACCATTCAAGCGGCAGTCACTTCCTTCCAGTGGCACAGATG-TAA  179
        ** **  * ** ** ** ** **  * **  * ** ** ** ****** *   **   **

tetM    GGTGAACATCATAGACACGCCAGGACATATGGATTTTTTAGCAGAAGTATATCGTTCATT  239
tetO    AGTCAACATTATAGATACGCCAGGCCATATGGATTTTTTGGCGGAAGTATACCGTTCTTT  239
tetW    AGTTAACATTGTGGATACGCCCGGCCACATGGATTTTTTGGCGGAGGTGTACCGCTCTTT  239
         ** *****  * ** ***** ** ** ********** ** ** ** ** ** ** **

tetM    ATCAGTATTAGATGGGGCAATTCTACTGATTTCTGCAAAAGATGGCGTACAAGCACAAAC  299
tetO    ATCCGTATTAGACGGAGCAGTATTATTAGTTTCTGCAAAGGATGGCATACAGGCACAGAC  299
tetW    GGCTGTTTTAGATGGGGCCATCTTGGTGATCTCCGCTAAAGATGGCGTGCAGGCCCAGAC  299
          * ** ***** ** **  *  *  *  * ** ** ** ****** * ** ** ** **

tetM    TCGTATATTGTTTCATGCACTTAGGAAAATAGGTATTCCCACAATCTTTTTTATCAATAA  359
tetO    CCGTATACTGTTTCATGCACTACAGACAATGAAGATTCCGACAATTTTTTTCATCAATAA  359
tetW    CCGTATTCTGTTCCATGCCCTGCGGAAAATGAACATTCCCACCGTTATCTTTATCAACAA  359
         *****  **** ***** **   ** ***    ***** ** *  * ** ***** **

tetM    GATTGACCAAAATGGAATTGATTTATCAACGGTTTATCAGGATATTAAAGAGAAACTTTC  419
tetO    AATTGACCAAGAGGGGATTGATTTGCCAATGGTATATCGAGAAATGAAAGCAAAGCTTTC  419
tetW    GATCGACCAGGCTGGCGTTGATTTGCAGAGCGTGGTTCAGTCTGTTCGGGATAAGCTCTC  419
         ** *****     **  *******    *  **   **          *   *  ** ** **

tetM    TGCGGAAATTGTAATCAAACAGAAGGTAGAACTGCATC  457
tetO    TTCGGAAATTATAGTGAAGCAAAAGGTTGGGCAGCAT-  456
tetW    CGCCGATATTATCATCAAGCAGACGGTGTCGCTGTCCC  457
          * ** *** *  * ** ** * ***    * *
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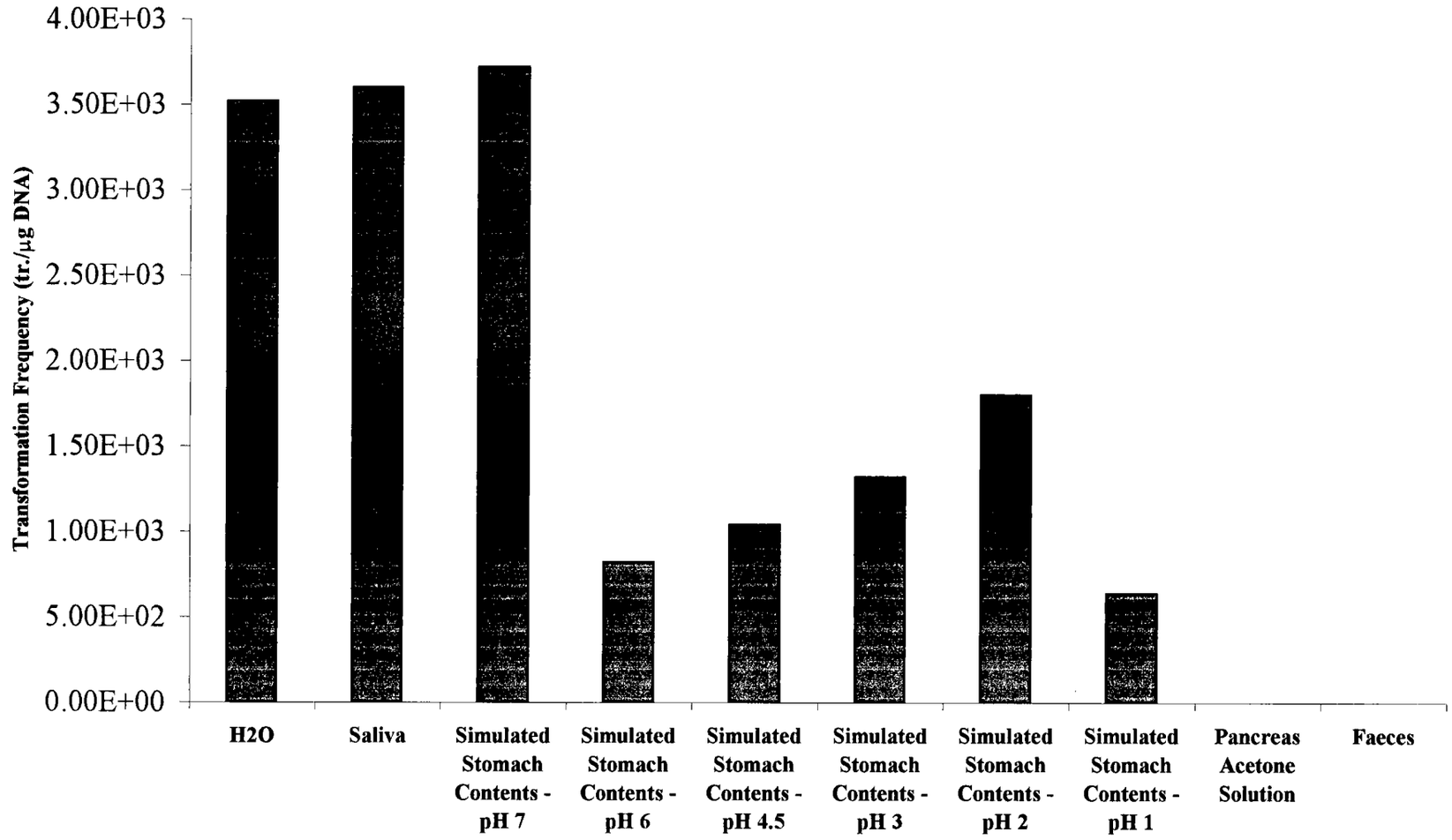
Figure 6: Effect of simulated gut contents on transformation of competent cells of *S. gordonii* DL1/Tn*916* with pKPSPgfp-int



distilled water as positive controls. No transformants were detected in the presence of simulated small intestine or colon contents, but transformants were detected in the presence of simulated stomach contaents at all pH's tested. Transformation was also attempted in the presence of a sample from a human colon-simulating fermentor, but no transformants were detected. Transformation of *S. gordonii* DL1 in the presence of simulated stomach contents, together with the fact that streptococci can be isolated from stomach contents (17) means that the possibility of natural transformation of bacteria in the human stomach cannot be ruled out.

Competence induction of *S. gordonii* DL1 and *S. gordonii* DL1/Tn*916* was attempted in the presence of simulated stomach contents (saliva acidified with 5M HCl), over a pH range of 1-7, simulated small intestinal contents (pancreas acetone solution) and simulated colonic contents (10% (w/v) human faecal slurry), but no transformation was detected using pVACMC1 and pKPSPgfp-int, respectively.

These results indicate that transformation is unlikely to make a significant contribution to horizontal gene transfer in the small intestine and colon, and that transformation in the stomach would only be possible if cells that are already competent enter the stomach.

Transformation of the human pathogen *Streptococcus bovis* NCTC11436

Streptococcus bovis is a facultative anaerobe commonly found in the of the rumen of cattle and sheep. *S. bovis* can also be isolated from the human gastro-intestinal tract where it can be the causative agent of a number of infectious diseases, including inflammatory bowel disease, intestinal cancer(3;20), meningitis(5), infective endocarditis, and bacteremias. Of major medical concern is the potential for pathogenic bacteria to acquire heterologous DNA by horizontal gene transfer, leading to a possible increase in pathogenicity, pathogen persistence, or resistance to a greater spectrum of antibiotics. The role of conjugation in horizontal gene transfer is well established, e.g. transfer of pathogenicity islands, but the role of transformation in pathogenicity has received relatively little attention.

S. bovis NCTC11436 was originally isolated from a blood culture from a Paris hospital in 1972 (4). Before attempting transformation of *S. bovis* NCTC11436 we established the erythromycin and tetracycline resistance profile of the strain, as these would be the selectable markers used in the transforming DNA. *S. bovis* NCTC11436 showed no resistance to erythromycin, but did show resistance to tetracycline at

concentrations up to 10 μg/ml (the concentration used to select for pAM120 transformation). By the use of PCR and sequencing we were able to establish that *S. bovis* NCTC11436 carries a Tn*916*/Tn*1545*-like conjugative transposon containing the *tet*(M) tetracycline resistance gene which was mobilisable (i.e. gene transfer by conjugation) from *S. bovis* NCTC11436 to a rifampicin resistant mutant of *S. gordonii* DL1 (conjugation frequency 1.2×10^{-7} per recipient).

Attempts were made to transform *S. bovis* NCTC11436 using the same protocol used to transform *S. gordonii* DL1 (14). Initial transformations were carried out with pKPSPgfp-int as we already knew that *S. bovis* NCTC11436 contained *tet*(M) within the chromosome and this plasmid gave the highest transformation frequencies when used to transform *S. gordonii* DL1/Tn*916* (15). Cells of *S. bovis* NCTC11436 were grown in BHI broths supplemented with 10% (v/v) heat-inactivated horse serum and samples were taken every hour to monitor growth and transformation by plating on BHI agar +/- 10 μg/ml erythromycin, respectively (Figure 7). As can be seen competence develops in early exponential phase and reaches an optimum efficiency after 6 hours that correlates with mid-exponential phase. Therefore, this pathogenic human *S. bovis* isolate is naturally transformable *in vitro*. It was also possible to transform *S. bovis* NCTC11436 with pVACMC1, but not pKPSPgfp-int(O) or pKPSPgfp-int(W) (Table 4).

Transformation of competent cells of *S. bovis* NCTC11436 was attempted in the presence of a range of different compounds (Figure 8). This demonstrates that *S. bovis* NCTC11436 can be transformed in the presence of filter-sterilised human saliva, but not a 10% (w/v) human faecal slurry. This experiment was repeated with simulated small intestinal contents and simulated stomach contents as described above for *S. gordonii* DL1, but no transformants were detected. Therefore, transformation of this human strain is unlikely to occur at any site in the gastro-intestinal tract other than the mouth.

We attempted to induce *S. bovis* NCTC11436 to develop competence with a range of different compounds other than the usual inducer, heat-inactivated horse serum. The effect of the different inducers on growth was also determined. From the results presented in Figure 9 it is clear that *S. bovis* NCTC11436 will develop competence in the absence of any apparent competence inducer (sterile distilled water) and that competence induction using only filter-sterilised human saliva

Figure 7. Competence development and transformation of *S. bovis* NCTC11436 with pKPSPgfp-int during exponential growth

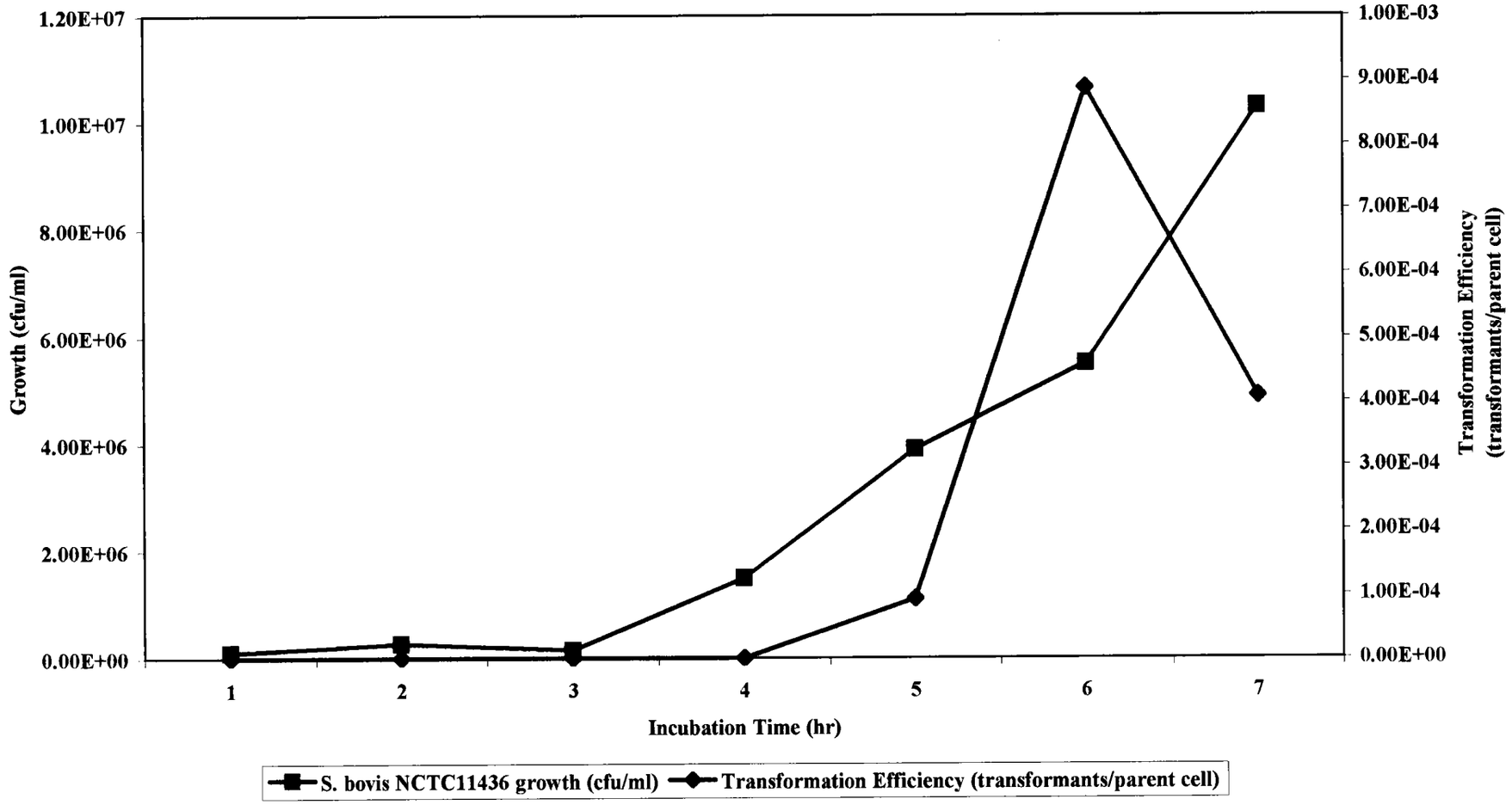


Table 4. Transformation of *Strpetococcus bovis* NCTC11436

DNA	Transformation Frequency (trans/μg DNA)	Transformation Efficiency (trans/parent cell)
pVACMC1	4.96×10^{2}	4.13×10^{-5}
pKPSPgfp-int	4.88×10^{3}	4.08×10^{-4}
pKPSPgfp-int(O)	0	0
pKPSPgfp-int(W)	0	0

Figure 8. Effect of the Presence of Different Compounds on the Transformation of *S. bovis* NCTC11436

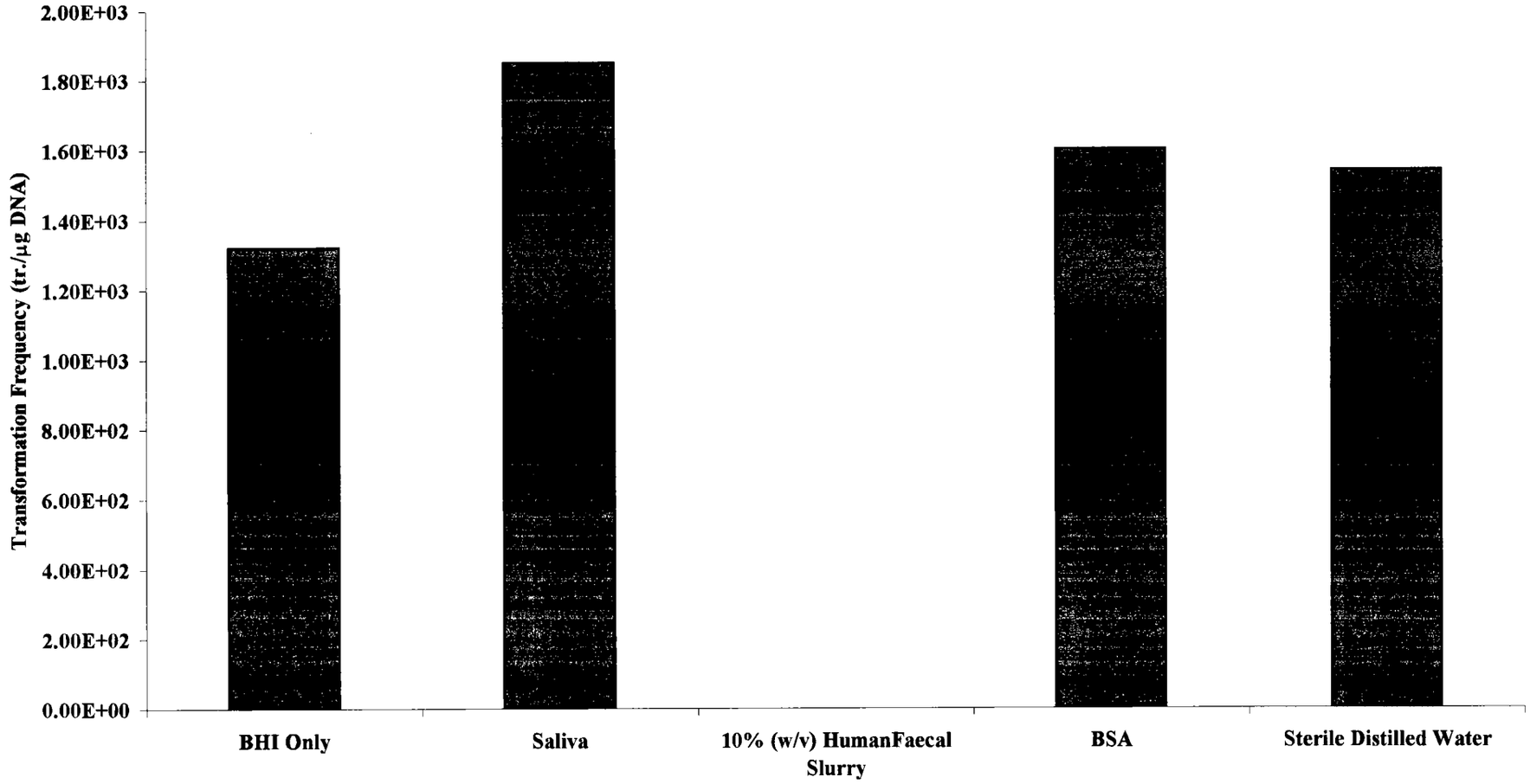
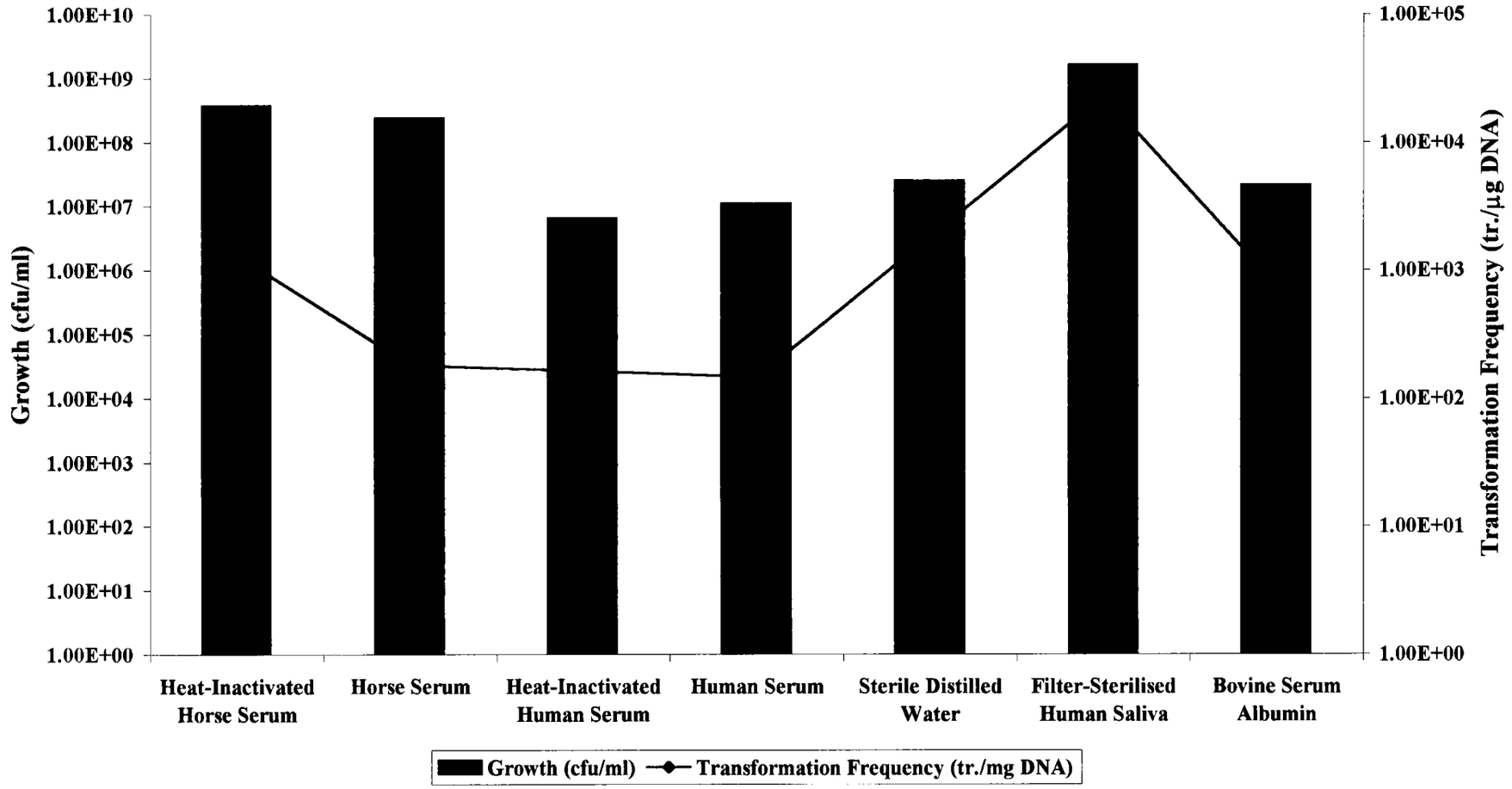


Figure 9. Effect of Competence Inducers on the Growth and Transformation Frequency of *S. bovis* NCTC11436



increases the transformation frequency of *S. bovis* NCTC11436 by 12.8-fold. No competence induction was observed when simulated stomach contents, pH range of 1-5, simulated small intestinal contents (pancreas acetone solution) and simulated colonic contents (10% (w/v) human faecal slurry) were used as competence inducers. It is possible that the presence of these compounds rapidly degrades the transforming DNA rather than inhibiting competence development. This pattern of competence induction (Figure 9) closely resembles that of the rumen strain *S. bovis* JB1 (13), rather than that of the human isolate, but different species, *S. gordonii* DL1 (14).

Therefore, transformation of a human pathogenic strain of *S. bovis* is possible under conditions simulating the oral cavity and, in common with the rumen strain *S. bovis* JB1, no inducer of competence development seems to be required.

Effect of food and foodstuffs on transformation of human streptococci

The effect of gut contents on streptococcal transformation has already been assessed (see above), but in the context of the fate of GM DNA it is also important to assess the role that food and food components are likely to have on DNA survival and transformation. Previous work had looked at the effect of different foods on the survival of pVACMC1 added to the food, using competitive PCR. This approach had lead to inconclusive results, possibly due to difficulties in extracting DNA after its addition, but the overall picture indicated that the foods tested lead to an increase in DNA degradation. As demonstrated previously (14), transformation can occur with DNA that has been exposed to saliva, even after the DNA appears degraded. Therefore, we examined the transformation of *S. gordonii* DL1 with pVACMC1 and *S. gordonii* DL1/Tn*916* & *S. bovis* NCTC11436 with pKPSPgfp-int in the presence of different foods and food components to determine whether transformation could occur.

Three foodstuffs were chosen to examine their effect on transformation, both in the presence and absence of saliva. Ball-milled wheat straw (0.1 g/ml BMWS) was chosen as a representative of complex carbohydrates, bovine serum albumin (0.1 g/ml BSA) was chosen to represent a food rich in protein and full-fat milk (10% (v/v) milk) as a food rich in both protein and fat. When designing this experiment we also had to take into account the methods used for transformation, so solid foods, or those that could not remain in a fine suspension or were immiscible (e.g. cooking oil) in a shaking water bath could not be used. Modification of experimental design might

facilitate examination of other foods, but this was not attempted here. The results (Figure 10) indicate that the transformation frequency of *S. gordonii* DL1/Tn*916* is slightly inhibited by the presence of BMWS and BSA and reduced by ~10-fold in the presence of milk, when compared with no additives. When *S. gordonii* DL1/Tn*916* was transformed in the presence of saliva transformation frequencies were reduced 22-fold, but when BSA was present there was a 2.5 times increase in transformation frequency. When milk was present there was a 50% reduction in transformation frequency and when BMWS was present no transformation was observed. These results indicate that saliva has a more significant effect on DNA survival and transformation than foodstuffs alone, but that the presence of foodstuffs in saliva may influence transformation, although this effect is likely to depend significantly on the content of the food.

The effect of the same foodstuffs on competence development of *S. gordonii* DL1 and *S. bovis* NCTC11436 was also examined (Figure 11). BSA induces competence development in both *S. gordonii* DL1 to give similar transformation frequencies to those observed when heat-inactivated horse serum is used (+ve control). This is perhaps unsurprising given that both additives contain serum proteins, albeit from different animals. Although BSA was not heat-inactivated (56°C for 30 min) prior to use in the same way that horse serum had been previous evidence has shown that heat-inactivation was not essential for induction of competence, but did leads to an increase in transformant numbers (14). Ball-milled wheat straw (BMWS) did not induce competence development in *S. gordonii* DL1. As shown in Figure 9 for *S. bovis* NCTC11436 and for *S. bovis* JB1 (13) this species of streptococcus does not seem to require any competence inducer. BMWS did not inhibit competence development for *S. bovis* NCTC11436. On the other hand, milk (when used as a possible competence inducer) resulted in no transformation and little or no growth of either strain over the 4-5 hour period.

A range of food components were chosen to determine their effect on transformation, both in the presence and absence of saliva (Figure 12). Xanthan gum, guar gum and chitosan were chosen as examples of carbohydrates and spermine was chosen as an example of a polyamine. Proteins had already been assessed (BSA and HI-HS) and miscibility problems associated with fats have already been mentioned and therefore fats were not used. The effect of xanthan gum and guar gum (30 mg/ml) on transformation is difficult to determine because at these concentrations there is a

Figure 10. Effect of the presence of foodstuffs and saliva on the transformation frequency of competent cells of *S. gordonii* DL1/Tn*916* & *S. bovis* NCTC11436

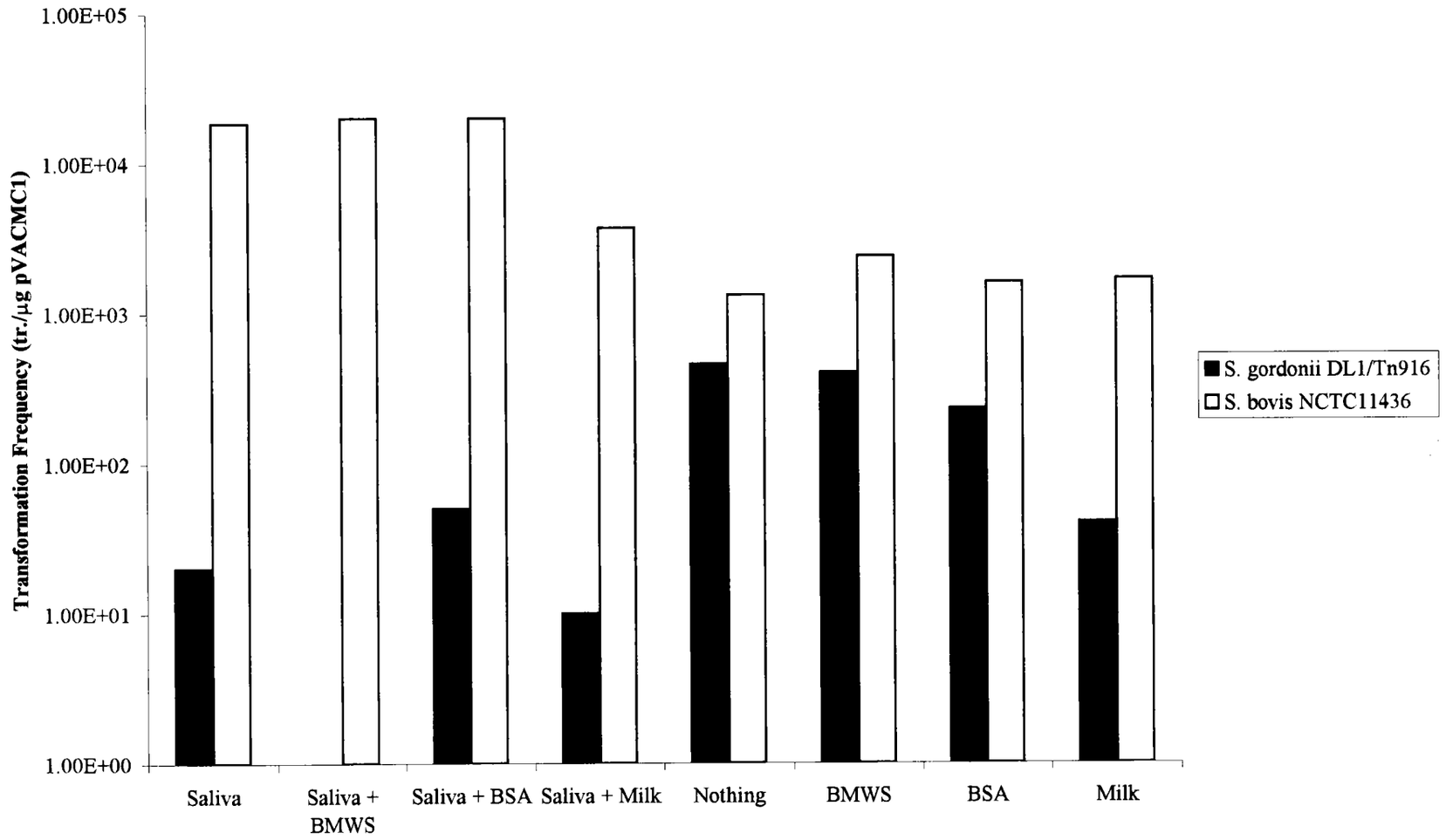


Figure 11. Effect of foodstuffs on the induction of competence and transformation of *S. gordonii* DL1 and *S. bovis* NCTC11436

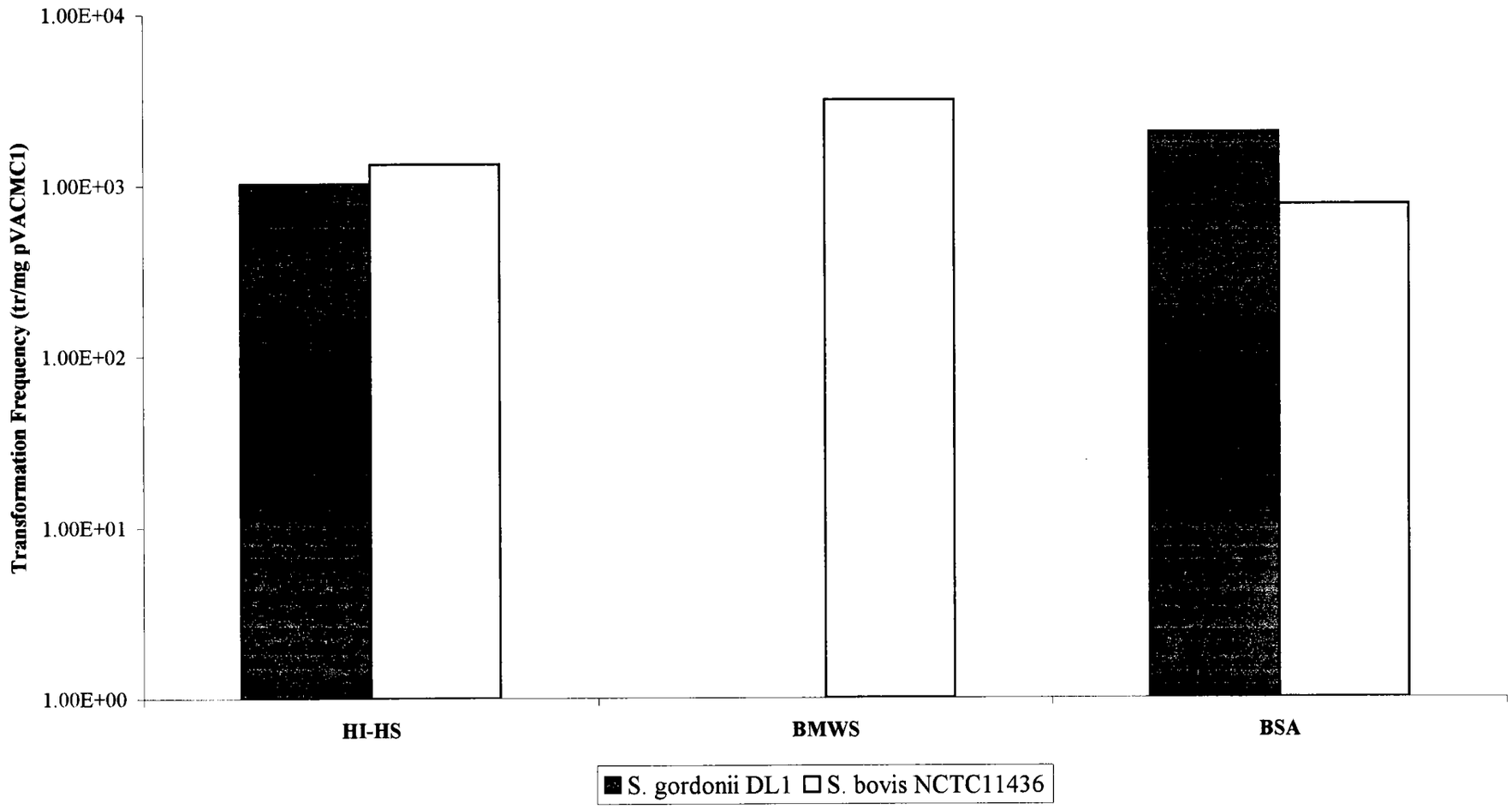
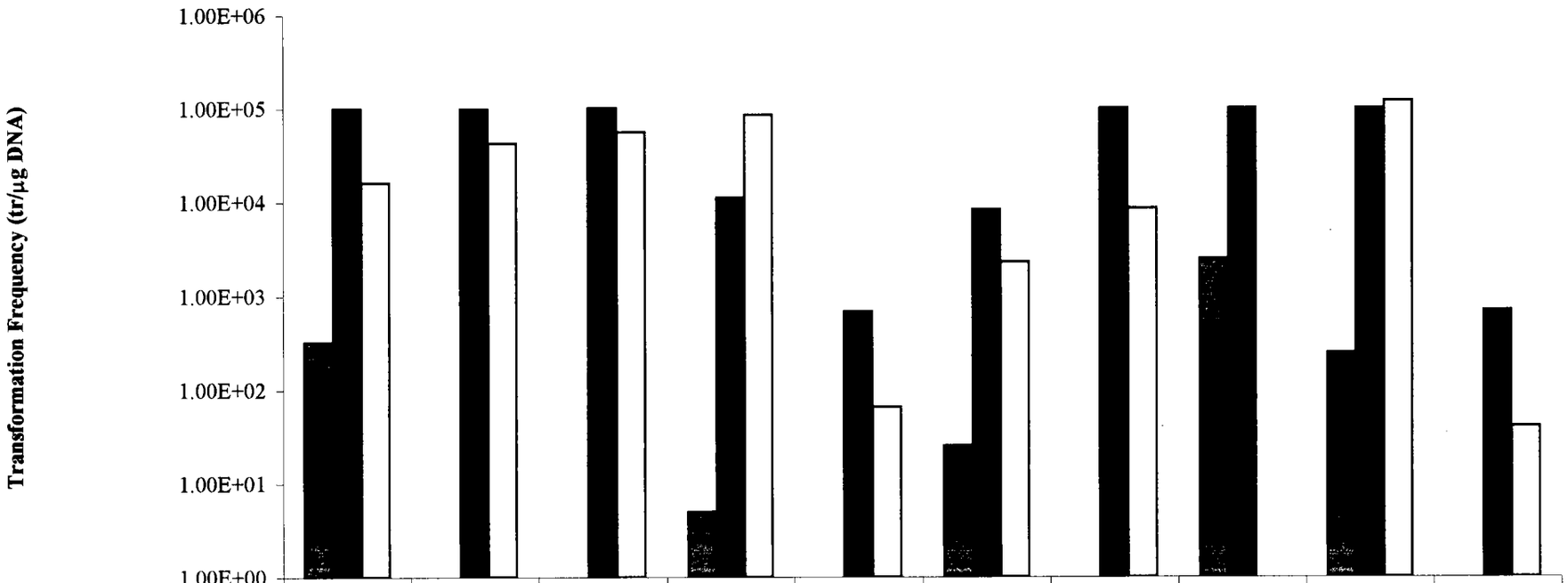


Figure 12. Effect of the presence of food components on the transformation of S. gordonii DL1, S. gordonii DL1/Tn916 & S. bovis NCTC11436



	No Additive	Xanthan Gum	Guar Gum	Chitosan	Spermine	Saliva	Saliva + Xanthan Gum	Saliva + Guar Gum	Saliva + Chitosan	Saliva + Spermine
S. gordonii DL1	3.20E+02	0.00E+00		5.00E+00	0.00E+00	2.50E+01		2.48E+03	2.45E+02	0.00E+00
S. gordonii DL1/Tn916	1.00E+05	9.94E+04	1.01E+05	1.11E+04	6.80E+02	8.40E+03	1.00E+05	1.00E+05	1.00E+05	7.00E+02
S. bovis NCTC11436	1.61E+04	4.29E+04	5.63E+04	8.60E+04	6.50E+01	2.30E+03	8.56E+03		1.20E+05	4.00E+01

S. gordonii DL1 S. gordonii DL1/Tn916 S. bovis NCTC11436

marked increase in viscosity of the cell suspensions. Therefore it is difficult to compare these results to those in the absence of xanthan or guar gum. However, it appears that both xanthan and guar gum have relatively little effect on transformation.

Chitosan (deacetylated chitin) is a cationic natural polysaccharide that can form polyelectrolyte complexes with DNA, thereby neutralising the DNA. The concentration used in this transformation experiment (30 mg/ml) should be sufficient to bind the DNA added to the transformation (1 μg). However, it is clear that when bound to chitosan the DNA remains available for transformation. When the chitosan transformation was carried out in the presence of saliva transformation frequencies were higher for *S. gordonii* DL1 and *S. bovis* NCTC11436, whereas they remained the same for *S. gordonii* DL1/Tn*916*. Therefore, chitosan may protect DNA from nucleolytic degradation while keeping the DNA available for transformation. This phenomenon has also been observed for the transformation of *Bacillus subtilis* with DNA bound to montmorillonite clays that is resistant to nucleolytic degradation (9). Spermine is a polyamine that can rapidly precipitate DNA (10 mM spermine can precipitate 20 μg calf thymus DNA in 15 minutes) (8). In this experiment 30 mg/ml spermine was added to competent cells followed by 1 μg transforming DNA. Therefore, the spermine should be able to precipitate the free DNA. However, transformation of *S. gordonii* DL1/Tn*916* and *S. bovis* NCTC11436 did occur in the presence of spermine, so it is clear that either complete precipitation does not occur, competent streptococci can take up the DNA faster than spermine can precipitate it or once precipitated the DNA remains available for transformation. Results are very similar in both the presence and absence of saliva indicating that spermine may protect DNA from the nucleolytic effects of saliva.

The results presented in this section are too preliminary to draw concrete conclusions. It is probable that the effect on transformation of every food and food component, both alone and mixed with others, would have to be assessed before any meaningful conclusions could be drawn. However, the increase in oral contents during the ingestion of food, the large spatial separation between most foods and the oral microflora and the structural nature of the food itself means that these factors probably contribute more significantly to the potential for transformation than the interaction between food, saliva and competent bacteria (if present).

Transformation of a Human Gut *E. coli* Isolate

A selection of 10 human gut *E. coli* isolates were screened for ampicillin resistance and only one was sensitive. This sensitive strain was used for the subsequent transformation experiments. Transformation was carried out in the absence of the traditional heat-shock treatment but in the presence of different compounds as described in Table 5 using the method of Baur, *et al.*, (1). Transformation of this strain was possible in the presence of $CaCl_2$ concentrations of 5 & 10 mM. When transformations were attempted in the presence of saliva no transformants were detected, but the concentration of $CaCl_2$ in saliva is low (1.5 mM). When the $CaCl_2$ concentration was increased by the addition of semi-skimmed milk (19 mM final concentration) still no transformants were observed. However, this may be due to increased rates of DNA degradation as we had previously shown that DNA degradation in saliva is rapid and the degradation is increased by semi-skimmed milk. When the $CaCl_2$ concentration was increased (10 mM $CaCl_2$ final concentration) large amorphous colonies were observed after overnight growth on agar plates containing ampicillin. These colonies could not be sub-cultured on medium containing ampicillin and we therefore have no evidence that they were transformants. The experiment was repeated a four times but no clear evidence of transformation of *E. coli* could be detected.

Transformation of *Helicobacter pylori*

Transformation of *H. pylori* was attempted following the method of E. El Omar (personal communication). Four human *H. pylori* strains were grown on Columbia agar plates with 5% defibrinated sheep blood. At all stages of the experiment *H. pylori* was sub-cultured at 37°C under microaerophilic conditions. A complete plate of cells was harvested and centrifuged at 13,000 rpm for 2 minute and gently resuspended in 50 µl of supernatant. 1 µg of *Helicobacter pylori* shuttle vector pHel2 (7) was added to the microcentrifuge tube containing the *H. pylori* cells and this transformation mixture was placed in the centre of a 5% sheep's blood Columbia agar plate (no antibiotic at this stage) and incubated upright until the medium is dry, then inverted and incubated for 24 hours. After 24 hours growth, the central "blob" of culture was plated out onto two plates with 5 µg/ml chloramphenicol and incubated for 3 to 4 days. Despite repeated attempts no transformants were obtained under these

Table 5 - Transformation of a Commensal Human Gut *E. coli* Isolate

Competence Inducer	Transformation Frequency (trans/μg pUC18)
O-2 mM $CaCl_2$	0
5 mM $CaCl_2$	2.1×10^2
10 mM $CaCl_2$	5.1×10^4
>15 mM $CaCl_2$	0
Whole Saliva (1.5 mM $CaCl_2$)	0
Filter-sterilised Saliva (1.5 mM $CaCl_2$)	0
Filter-sterilised Saliva + 10 mM $CaCl_2$	??
Filter-sterilised Saliva + Semi-skimmed Milk (19 mM $CaCl_2$)	0

supposedly optimal *in vitro* conditions. Therefore, this experiment could not be conducted to test transformation of *H. pylori* under simulated stomach conditions.

Survival of GM Bacteria in Human Gut Fermentor Simulations

A human colon fermentor simulation was set up with an initial human faecal inoculum and run for 28 days. After 5 days to allow stabilisation the fermentor was inoculated with 1 x 10^9 cfu *L. lactis*/Tn*916/gfp* containing chromosomally integrated gfp gene. Further details of the fermentor system used and construction of the strains are described in Scott, et al, (2000) (19). Survival of the introduced strain was monitored over the next 7 days (Figure 13). Counts of *L. lactis* dropped rapidly (much faster than the washout rate), suggesting that the cells are being actively killed by other bacteria present in the fermentor (Figure 13). This is not surprising because *L. lactis* is normally found only transiently in the human gut and is unable to colonise (10). However, on day 7 there were still some Tc^R, Em^R cells carrying *gfp* present. These must represent either adaptation of the strain to survive under fermentor conditions, or possible transfer of Tn*916*::pKPSPgfp-int to a Gram positive coccoid bacterium better adapted to survive under human colon conditions. However, Tn*916* is not able to mobilise itself from *L. lactis* although sex-factor mediated transfer is found (2). The surviving cells were confirmed as the introduced strain by analysis of antibiotic resistance, fluorescence, microscopic analysis and comparison of protein profiles of cell extracts from the introduced and surviving bacteria after SDS-PAGE.

On day 7 the fermentor was inoculated with 1 x 10^9 cfu of *E. faecalis*/Tn*916/gfp* cells. *E. faecalis*/Tn*916/gfp* survived much better in the fermentor than the *L. lactis* strain. Loss of *E. faecalis*/Tn*916/gfp* from the fermentor paralleled the washout rate. *E. faecalis* is a common human gut bacterium, but this laboratory strain was unable to establish.

The interesting results obtained on survival of *L. lactis*/Tn*916/gfp* in the first fermentor run meant that the experiment was repeated and *L. lactis*Tn*916/gfp* survival was monitored for 38 days (Figure 14). From this experiment the surviving tail population was much clearer. When chromosomal DNA from samples of the surviving tail population were analysed using a primer designed to specifically amplify *L. lactis* (16) in combination with the eubacterial rP2 primer (21) and by SDS-PAGE total protein profiling, it was shown that the tail population was indeed *L.*

Figure 13 - Survival of *L. lactis*/Tn*916*/*gfp* and *E. faecalis*/Tn*916*/*gfp* in a Human Colon Fermentor

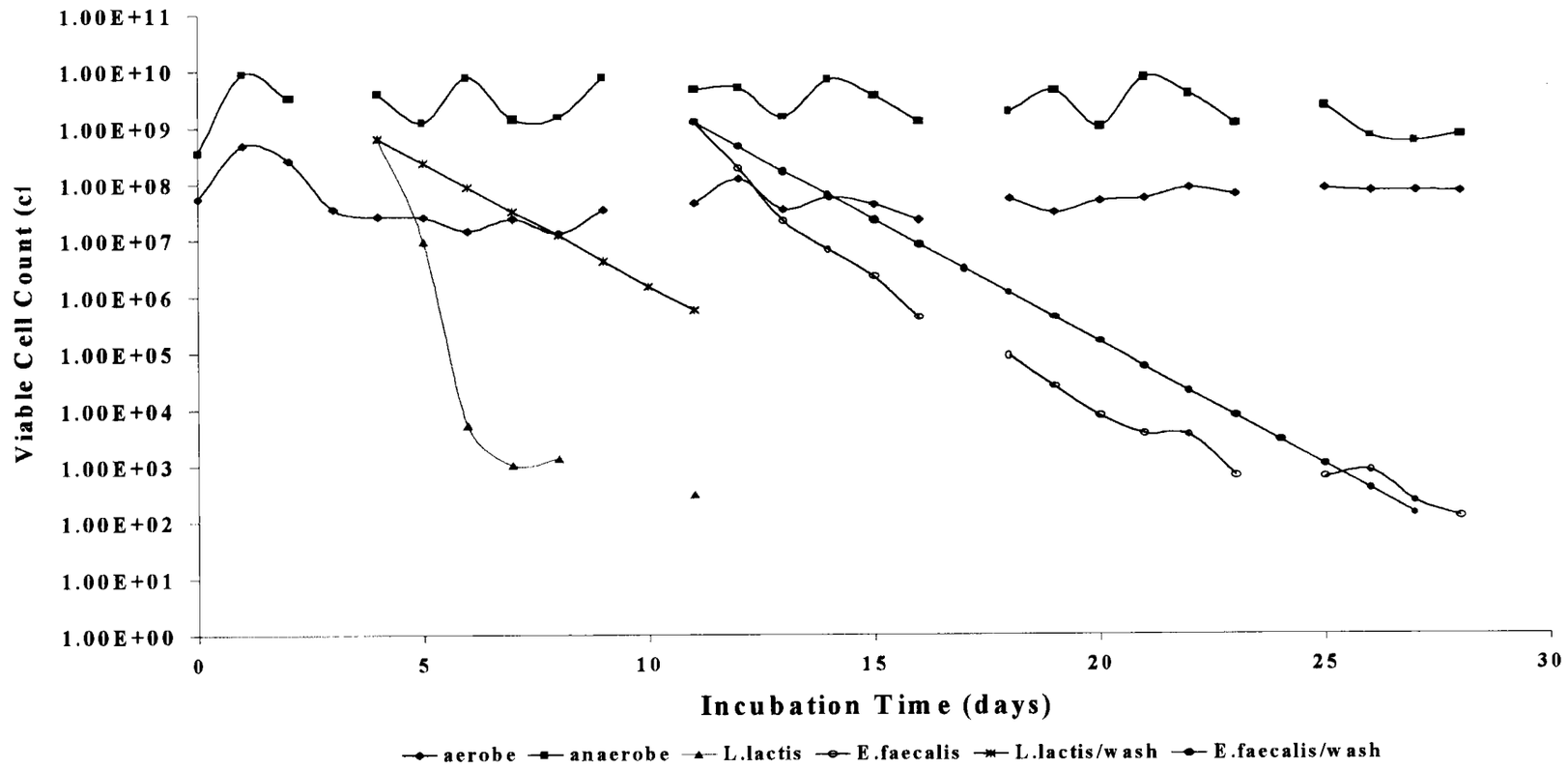
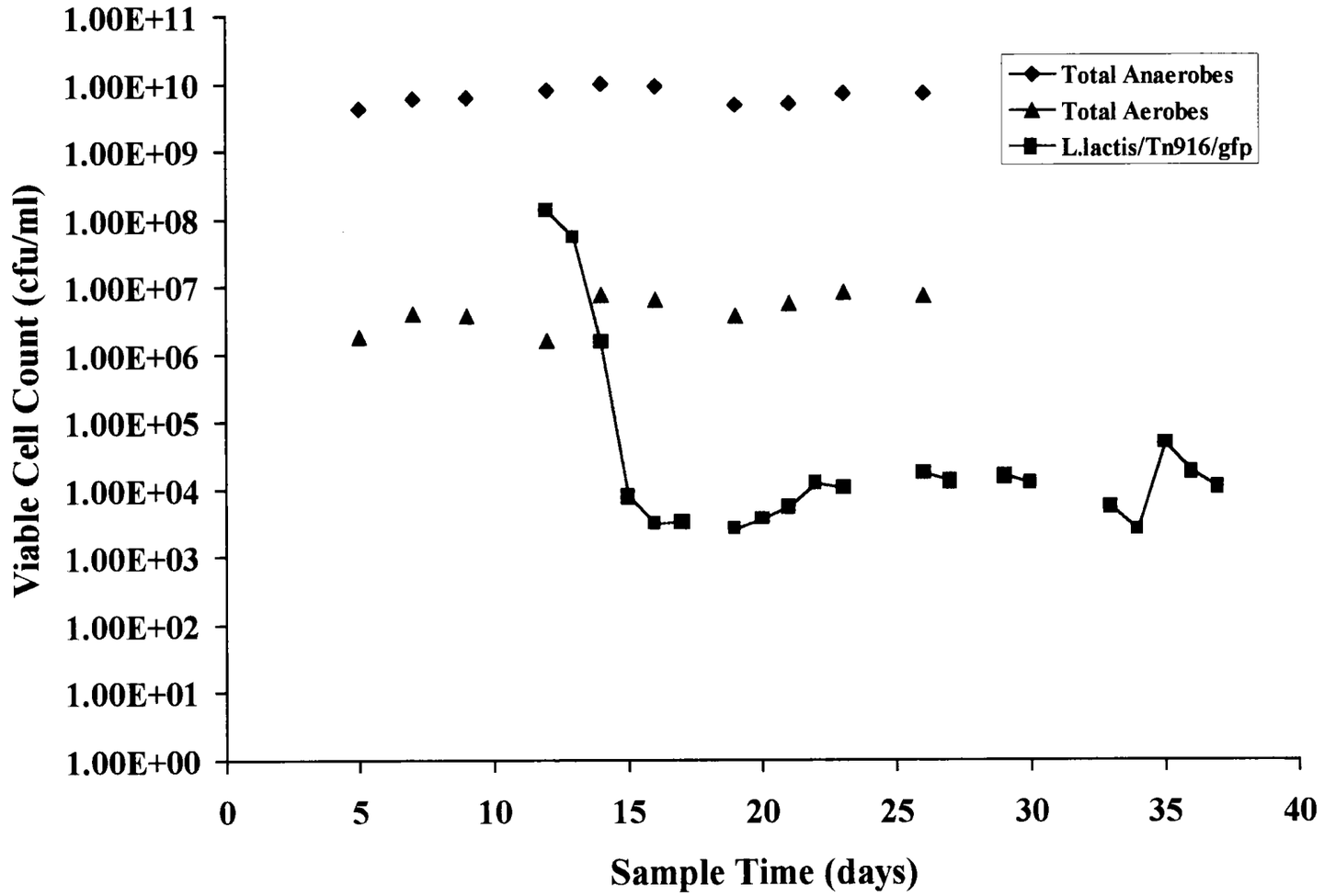


Figure 14 - Survival of *L. lactis*/Tn*916/gfp* in a Human Colon Fermentor



lactis and not another Gram positive coccus. This meant that the original strain had adapted to fermentor conditions.

Surviving bacteria from both fermentor runs (cultures 2 and 3 respectively) were compared directly with the original strain (culture 1) in a number of growth media, over a temperature range (25-45°C), over a pH range (pH 5.5-7.5) and in their ability to grow in the presence of short-chain fatty acids. In all cases growth of all 3 strains was identical as indicated in the representative graphs shown in Figure 14a and 14b, confirming that *L. lactis* Tn*916/gfp* had adapted to fermentor conditions, and that gene transfer had not been detected.

Survival of GM DNA and *E. faecalis*/Tn*916/gfp* in the Rat Gut

Two otherwise untreated hooded Lister rats were inoculated with 1 ml of 1.75 x 10^9 cfu *E. faecalis*/Tn*916/gfp* and the survival was monitored in rat faeces for 14 days. The inoculated strain was able to survive for at least 11 days in the rat gut (Figure 15) and represented 0.001% of the rat gut flora on day 11. Gene transfer events could have resulted in the transfer of an element conferring erythromycin resistance, but the high background population of erythromycin resistant bacteria in the rat gut (1-10%) meant that detection of gene transfer was not possible. The plasmid pKPSPsgfp was therefore modified to confer chloramphenicol resistance as well as erythromycin resistance (See sections below: Construction of the Shuttle Vector pKPSPsgfpCm^R & Examination of the potential for gene transfer in the rat gut), since there is very little background of chloramphenicol resistance in the rat gut. It may therefore be possible to detect gene transfer, if it is occurring, by the detection of chloramphenicol and erythromycin resistant colonies that are not resistant to rifampicin, as *E. faecalis*/Tn*916/gfp* is a rifampicin resistant mutant.

In the rat sacrificed after 24 hours *E. faecalis*/Tn*916/gfp* was found in all gut compartments isolated after dissection (both attached and luminal flora) (Figure 16). This may indicate colonisation of the rat gut by *E. faecalis*/Tn*916/gfp*, but it may show that *E. faecalis*/Tn*916/gfp* is only gradually eliminated from the rat gut and numbers are still high soon after inoculation. When the second rat was dissected after 14 days no *E. faecalis*/Tn*916/gfp* was found in any of the gut contents, showing that it had been completely eliminated.

Figure 14 a: Growth of *L. lactis* at pH=6.5

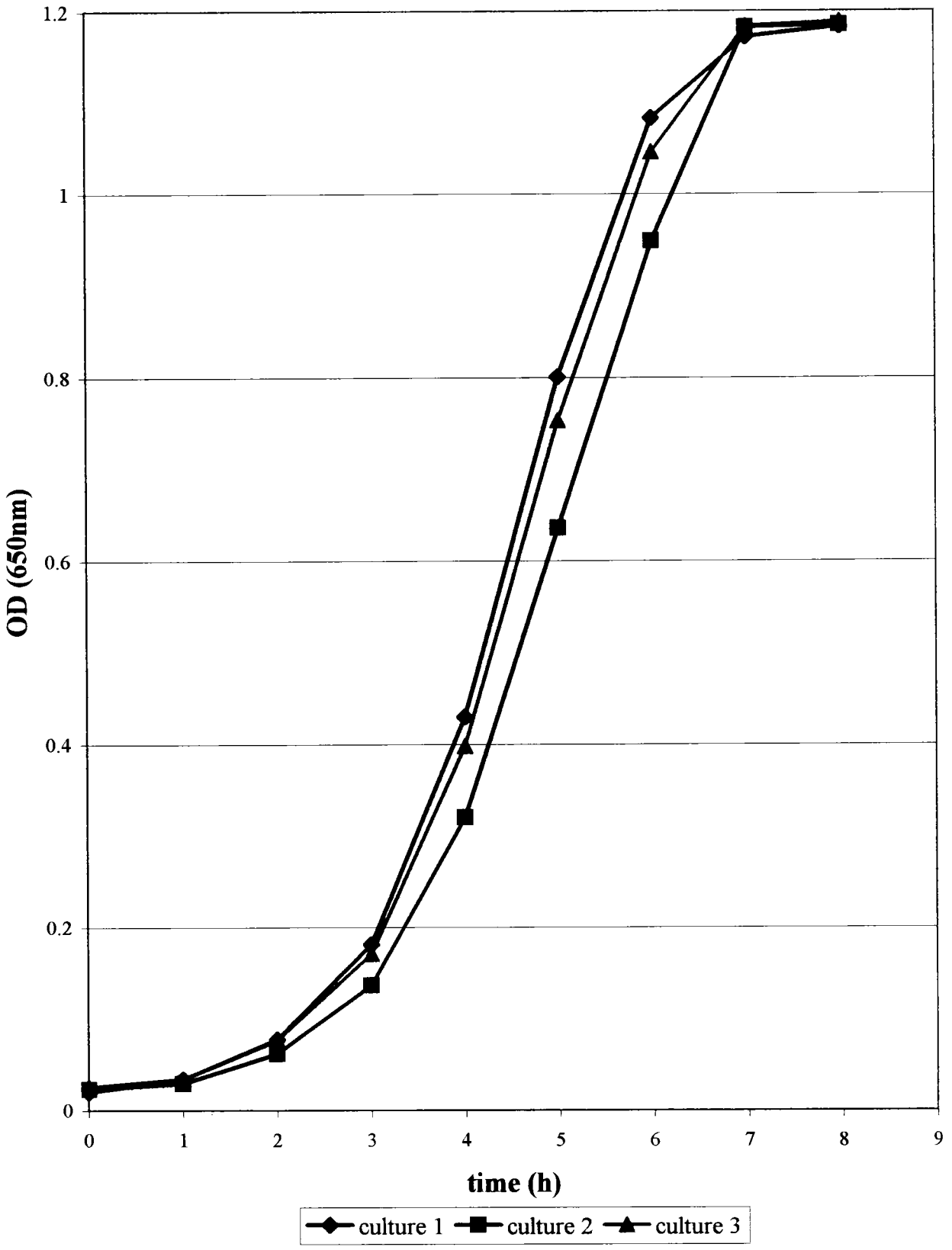


Figure 14b: Growth of L. lactis at 40°C

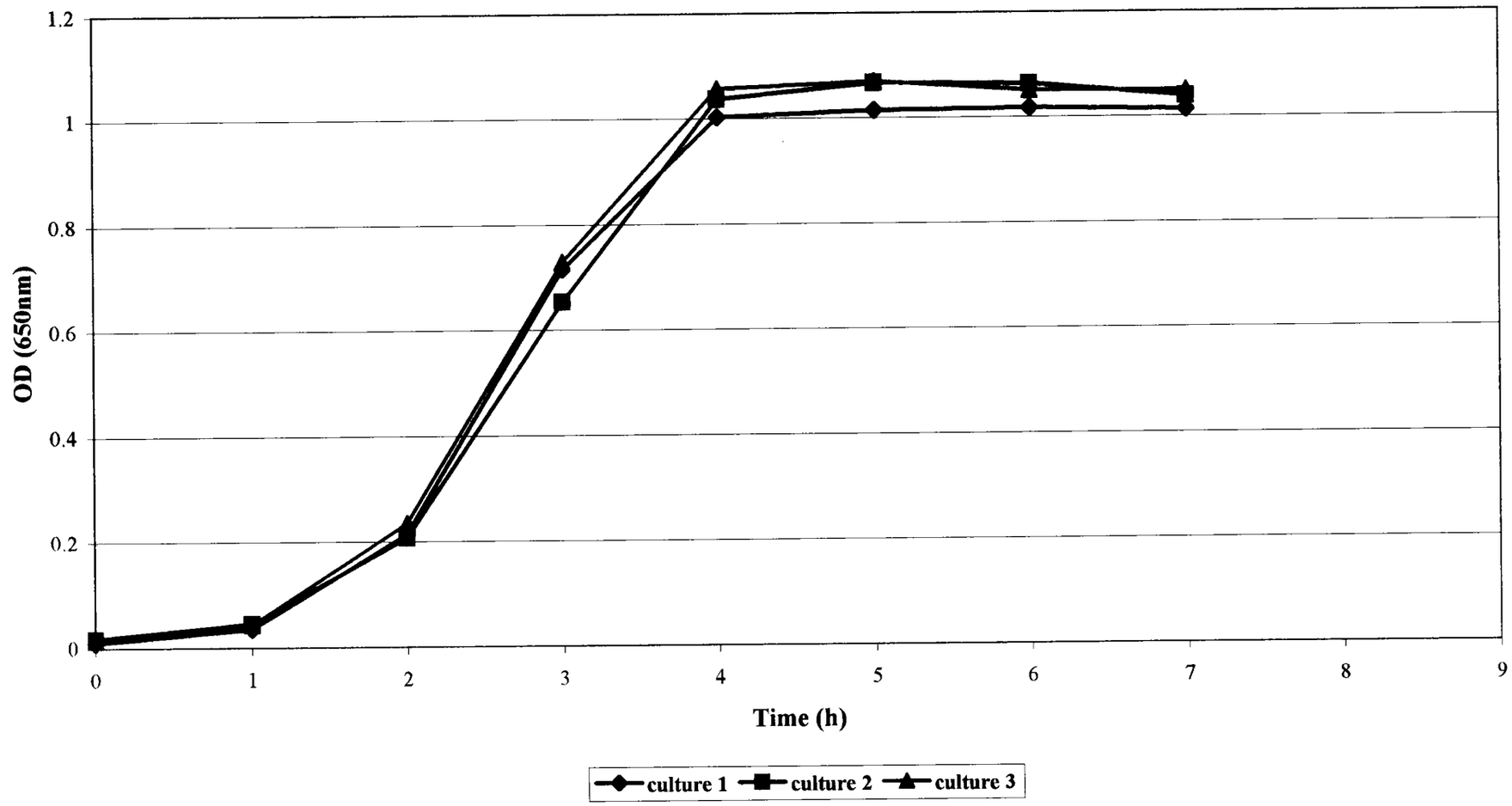


Figure 15 - Survival of *E. faecalis*/Tn*916*/*gfp* in the Rat Gut

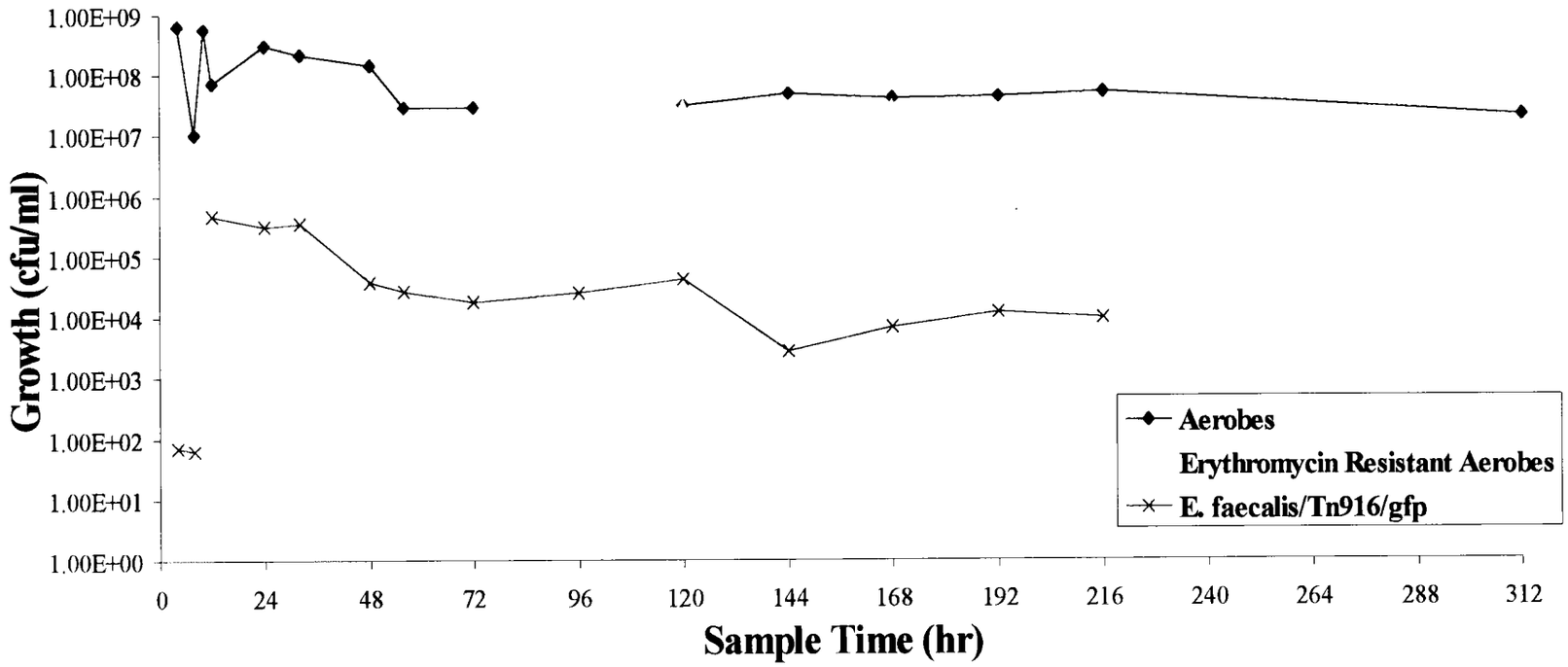
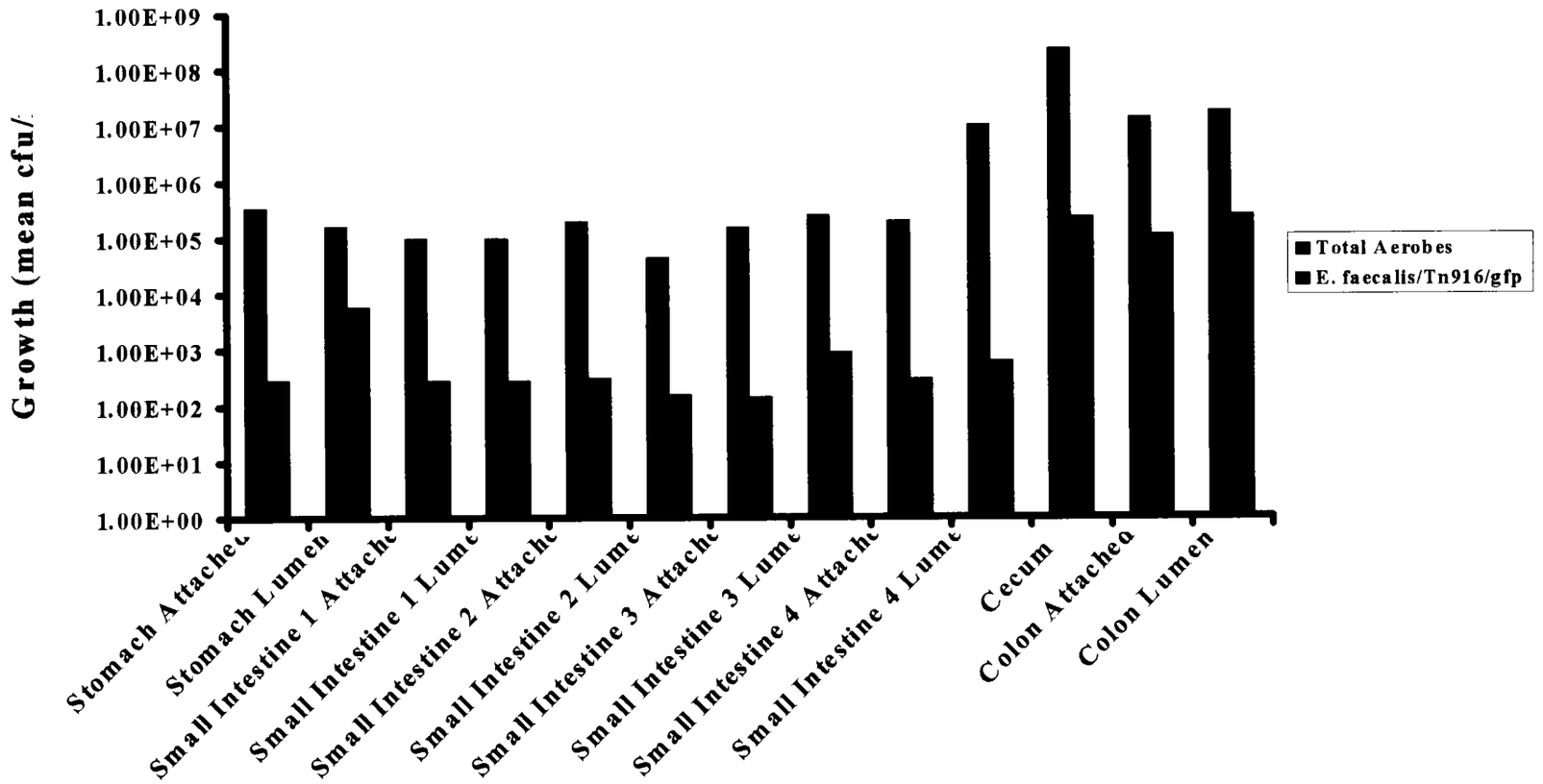


Figure 16 - Survival of *E. faecalis*/Tn*916/gfp* in the Rat Gut 24 Hours after Inoculation



A third rat was inoculated 150μg pKPSPsgfp in 1 ml TE buffer. Survival of DNA in rat faeces was monitored by PCR amplification of a fragment of the *gfp* gene from rat faeces and by Southern hybridisation using a probe based on a 610 bp fragment of the *gfp* gene for 14 days before the rat was sacrificed. No background amplification of the *gfp* gene was observed in rat faeces prior to dosing with pKPSPSgfp. *gfp* DNA was detectable by PCR and Southern hybridisation up to 79 hours after the rat was first inoculated with pKPSPsgfp. In most cases the expected 500 bp PCR product was observed, but in some cases a previously unseen 1 kb PCR product was observed. This therefore suggests significant survival of free DNA in a gut ecosystem, although DNA sequence was not obtained for the PCR products. DNA was extracted from rat faeces throughout the experiment and separated into three fractions (supernatant, microbial, debris) according to the protocol of Klijn and co-workers (10). Polymerase chain reaction to amplify a 610 bp fragment of the *gfp* gene (not present in rats not fed pKPSPsgfp) was carried out (Table 6). The *gfp* gene fragment was amplifiable for up to 8 hours in the supernatant fraction and for up to 32 hours in the microbial and debris fraction. Survival of free pKPSPsgfp DNA in the supernatant fraction for 8 hours was surprising as it was anticipated that DNA degradation would have occurred more rapidly. However, survival for up to 32 hours in the microbial fraction indicates that within this fraction the DNA has been protected from degradation. It is possible that protection of the DNA from degradation occurred because the DNA was taken up by members of the resident microflora by transformation, indicating that gene transfer may have occurred in the rat gut. However, because of the high background of erythromycin resistance in rat faeces we were unable to isolate transformants and therefore cannot confirm unambiguously that gene transfer has occurred.

Construction of the Shuttle Vector pKPSPsgfpCm^R

To facilitate detection of gene transfer pKPSPsgfp was modified to include a chloramphenicol resistance gene in addition to the erythromycin resistance gene by constructing the plasmid pKPSPsgfp-Cm^R (Figure 17). Primers were designed to amplify the complete chloramphenicol resistance gene, including 200 bp of regulatory upstream sequence, from plasmid pVA797. The primers included *Bam*H1 sites to facilitate cloning directly downstream of the *gfp* gene in the plasmid pKPSPsgfp.

Table 6: Survival of a 610 bp fragment of the *gfp* gene in the faeces of a rat fed 150 μg pKPSPsgfp

Sample Time (hours)	Supernatant	Microbial Fraction	Debris Fraction
0	-	-	-
8	+	+	+
24	-	+	+
32	-	+	+
48	-	-	-
56	-	-	-

Following ligation into pKPSPsgfp the structure of the resulting plasmid was confirmed by PCR and restriction digestion. The plasmid confers resistance to 5μg/ml chloramphenicol in *E. coli* and *S. gordonii* DL1, and 25 μg/ml chloramphenicol in *S. bovis* JB1 and *S. bovis* NCTC11436, when 10μg/ml erythromycin was also present. Transformants were also obtained when 5μg/ml chloramphenicol was used in combination with 50μg/ml erythromycin, but when 25μg/ml or 10μg/ml chloramphenicol was used in combination with 50μg/ml erythromycin no transformants were detected for all 4 strains tested. When 5μg/ml chloramphenicol was used for selection of background resistance in rat faeces 9.6 x 10^3 cfu/ml was observed. Microscopic analysis of resistant bacteria revealed the presence exclusively of Gram negative rods and coccobacilli. When 5μg/ml chloramphenicol was used in combination with 50μg/ml erythromycin there was a ~10% reduction in background resistance (8.58 x 10^3). When 50μg/ml erythromycin alone was used the background resistance was much higher 1.7 x 10^5 cfu/ml (~17-fold more resistance). When 50μg/ml erythromycin and 25 or 10μg/ml chloramphenicol were used no background resistance was observed when dilutions of rat faeces were spread on BHI agar containing these antibiotics. Despite some background resistance at low chloramphenicol concentrations pKPSPsgfpCm^R was used to examine gene transfer in the rat gut.

Examination of the Potential for Gene Transfer by Transformation in the Rat Gut

A second rat experiment was set up to examine the potential for gene transfer in the rat gut using the plasmid pKPSPsgfpCm^R in place of pKPSPsgfp. Four Hooded Lister rats were dosed with pKPSPsgfpCm^R as described above for pKPSPsgfp, but otherwise all experimental protocols remained the same. Rat 1 was sacrificed after 24 hours, rat 2 after 48 hours, rat 3 after 72 hours and rat 4 after 96 hours. Faecal samples were collected throughout this period and after sacrifice small intestinal, caecal and colonic contents (luminal and attached) were collected. All samples were treated aerobically, diluted accordingly with sterile Ringers solution and plated on Brain Heart Infusion agar containing chloramphenicol, and erythromycin where appropriate. In all cases colony counts represent the means of duplicate plates. Colony counts on non-selective media remained relatively constant throughout the experiment for all 4

rats (an example for rat 4 is shown in Figure 18), whereas the results for the different gut compartments showed some differences between rats (Table 7). Based on the results of background resistance of chloramphenicol in rat faeces and the level of chloramphenicol resistance conferred by pKPSPsgfpCm^R we decided to look for transformation on BHI agar containing the following antibiotic concentrations: 5μg/ml chloramphenicol, 10μg/ml chloramphenicol and 5μg/ml chloramphenicol + 50μg/ml erythromycin. The colony counts for faecal samples plated on BHI containing 10μg/ml chloramphenicol or 5μg/ml chloramphenicol + 50μg/ml erythromycin are presented in Table 8. When plates containing 5μg/ml chloramphenicol alone were used colony counts after pKPSPsgfpCm^R administration showed no statistically significant differences (p<0.05) between the background levels detected prior to pKPSPsgfpCm^R administration (data not shown). Colony counts from plates containing 10μg/ml chloramphenicol or 5μg/ml chloramphenicol + 50μg/ml erythromycin showed significant increases in numbers of resistant bacteria for all four rats. These results demonstrate the presence of resistant bacteria in the faeces of rats inoculated with pKPSPsgfpCm^R where no resistant bacteria were present previously. This may indicate that transformation of bacteria in the rat gut with pKPSPsgfpCm^R has occurred. A random sample of 24 colonies was picked from each of the resistance profiles (10μg/ml chloramphenicol and 5μg/ml chloramphenicol + 50μg/ml erythromycin) from each rat where resistant colonies were found (196 colonies in total) and sub-cultured on the same media. Fragments of colonies were Gram stained and examined microscopically. Both Gram negative and Gram positive bacteria were present and included rods, cocci and coccobacilli, with no given colony morphology dominating. Further colony fragments were screened by PCR from boilates (6) for the presence of a 720 bp fragment of the Cm^R gene present on pKPSPsgfpCm^R. In no cases was any PCR product detected indicating that none of the resistant colonies had been transformed to chloramphenicol resistance by pKPSPsgfpCm^R (data not shown). A second random sample of 48 colonies were sub-cultured into BHI broths containing the same antibiotics and grown overnight at 37°C. Plasmid DNA was isolated from these cultures and PCR's were carried out to detect the same fragment of the Cm^R gene, and a ~650 bp fragment of the *gfp* gene (18). Again no PCR products were detected indicating that none of the resistant colonies had been transformed to chloramphenicol resistance by pKPSPsgfpCm^R. The reason

Figure 17: **pKPSPsgfpCm^R**

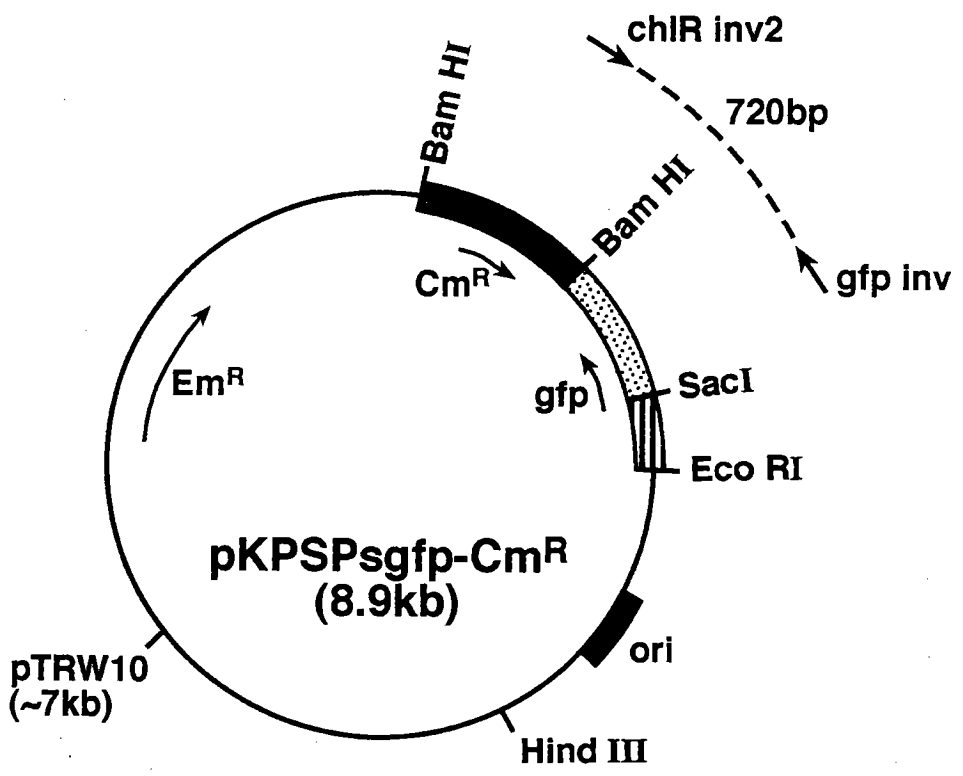


Figure 18: Aerobic growth of rat faecal bacteria (Rat 4) on BHI agar

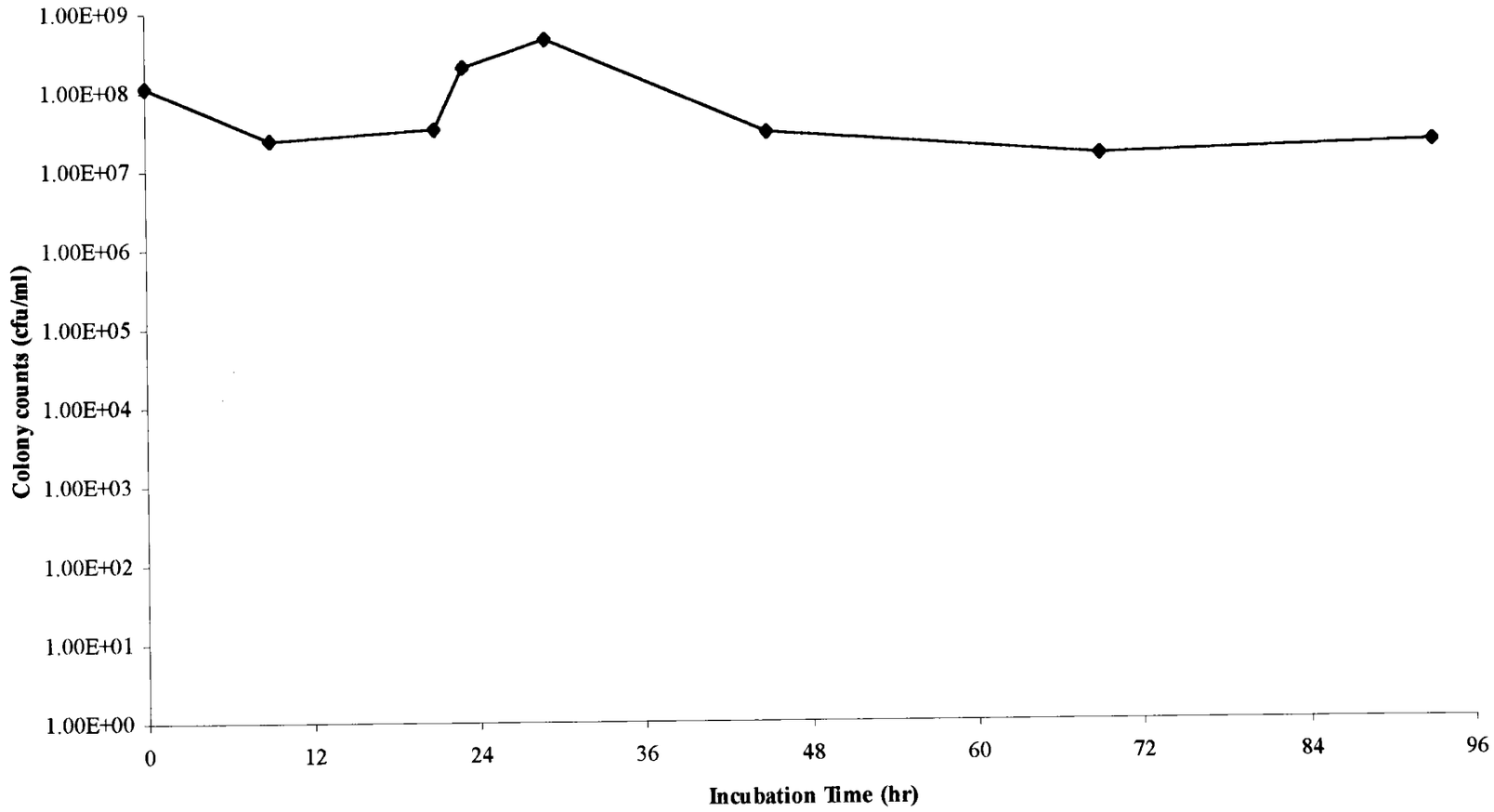


Table 7: Aerobic growth of rat gut content samples on BHI agar

	Rat 1	Rat 2	Rat 3	Rat 4
Small Intestine	2.88×10^9	1.70×10^8	1.49×10^8	2.81×10^7
Caecum	1.36×10^9	1.36×10^9	1.24×10^8	3.32×10^7
Colon	1.48×10^8	2.68×10^9	3.68×10^7	4.54×10^7

Table 8: **Antibiotic resistance profiles of rat faeces from rats dosed with 150 μg pKPSPgfpCmR**

	Rat 1		Rat 2		Rat 3		Rat 4	
Incubation Time (hr)	**BHIC(10)[3]**	**BHIC(5)E(50)[4]**	**BHIC(10)[1]**	**BHIC(5)E(50)[2]**	**BHIC(10)[1]**	**BHIC(5)E(50)[2]**	**BHIC(10)[1]**	**BHIC(5)E(50)[2]**
Uninoculated	0	0	0	0	0	0	0	0
9	NS[5]	NS[3]	NS[3]	NS[3]	NS[3]	NS[3]	0	0
21	0	2.00E+03	0	2.00E+03	2.00E+02	1.20E+04	5.00E+02	1.00E+03
23	**Sacrificed**		8.64E+04	1.70E+03	NS[3]	NS[3]	5.00E+03	6.08E+04
29			NS[3]	NS[3]	6.00E+02	0	5.00E+03	3.36E+04
45			0	0	0	0	0	0
69		**Sacrificed**	0	0	0	0	0	0
93					**Sacrificed**		5.00E+02	5.00E+02
96							**Sacrificed**	

[3] BHIC(10) – BHI agar + 10 μg/ml chloramphenicol

[4] BIHC(5)E(50) - BHI agar + 5 μg/ml chloramphenicol + 50 μg/ml erythromycin

[5] NS – No faecal sample produced

for the appearance of resistant colonies after pKPSPsgfpCm^R administration is therefore unclear.

After rats were sacrificed small intestinal, caecal and colonic contents (attached and luminal) were plated on the selective media described above (Figure 19 & 20). These results indicate that there are similar numbers of bacteria resistant to 5μg/ml chloramphenicol + 50μg/ml erythromycin in all 4 bacteria in the small intestine, caecum and colon (Figure 19), whereas the numbers of bacteria resistant to 10μg/ml chloramphenicol are very different between rats and within gut compartments (Figure 20). A random sample of 12 colonies were taken from plates resistant bacteria from each of the gut compartments in which colonies were isolated (i.e. 12 x 19 = 228) and sub-cultured on the same media. Fragments of colonies were Gram stained and examined microscopically. Both Gram negative and Gram positive bacteria were present and included rods, cocci and coccobacilli, with no given colony morphology dominating. Further colony fragments were screened by PCR from boilates (6) for the presence of a 720 bp fragment of the Cm^R gene present on pKPSPsgfpCm^R. In no cases was any PCR product detected indicating that none of the resistant colonies had been transformed to chloramphenicol resistance by pKPSPsgfpCm^R (data not shown).

All plates were examined microscopically for evidence of green fluorescence (due to expression of the *gfp* gene). Random strongly fluorescent colonies (25) were picked off the dilution plates and sub-cultured on BHI agar medium containing, either chloramphenicol (10 μg/ml) or 5 μg/ml chloramphenicol + erythromycin (50 μg/ml) All 25 colonies grew and were Gram negative rods. The 25 colonies were then sub-cultured onto agar medium containing kanamycin (100 μg/ml) that inhibits the growth of *E. coli* and all colonies grew. Both genomic and plasmid DNA was extracted from 10 colonies. DNA was then PCR amplified with the pKPSPsgfpCm^R specific primers to check authenticity of the transformants. However, no PCR products were obtained. Therefore, chloramphenicol resistance was probably due to background resistance and not gene transfer of the pKPSPsgfpCm^R plasmid and subsequent transformation.

DNA was extracted from faecal and tissue samples (small intestine, caecal, and colonic contents) using the QIAamp DNA stool mini kit, Qiagen, UK. Total DNA was PCR amplified using a primer set which specifically amplifies a 720 bp fragment of the pKPSPsgfpCm^R plasmid containing part of the *gfp* gene and part of the Cm^R

Figure 19: Distribution of Antibiotic Resistant Bacteria (5 mg/ml Cm + 50 mg/ml Em) in Rat Gut Compartments of Rats inoculated with pKPSPgfpCm^{R}

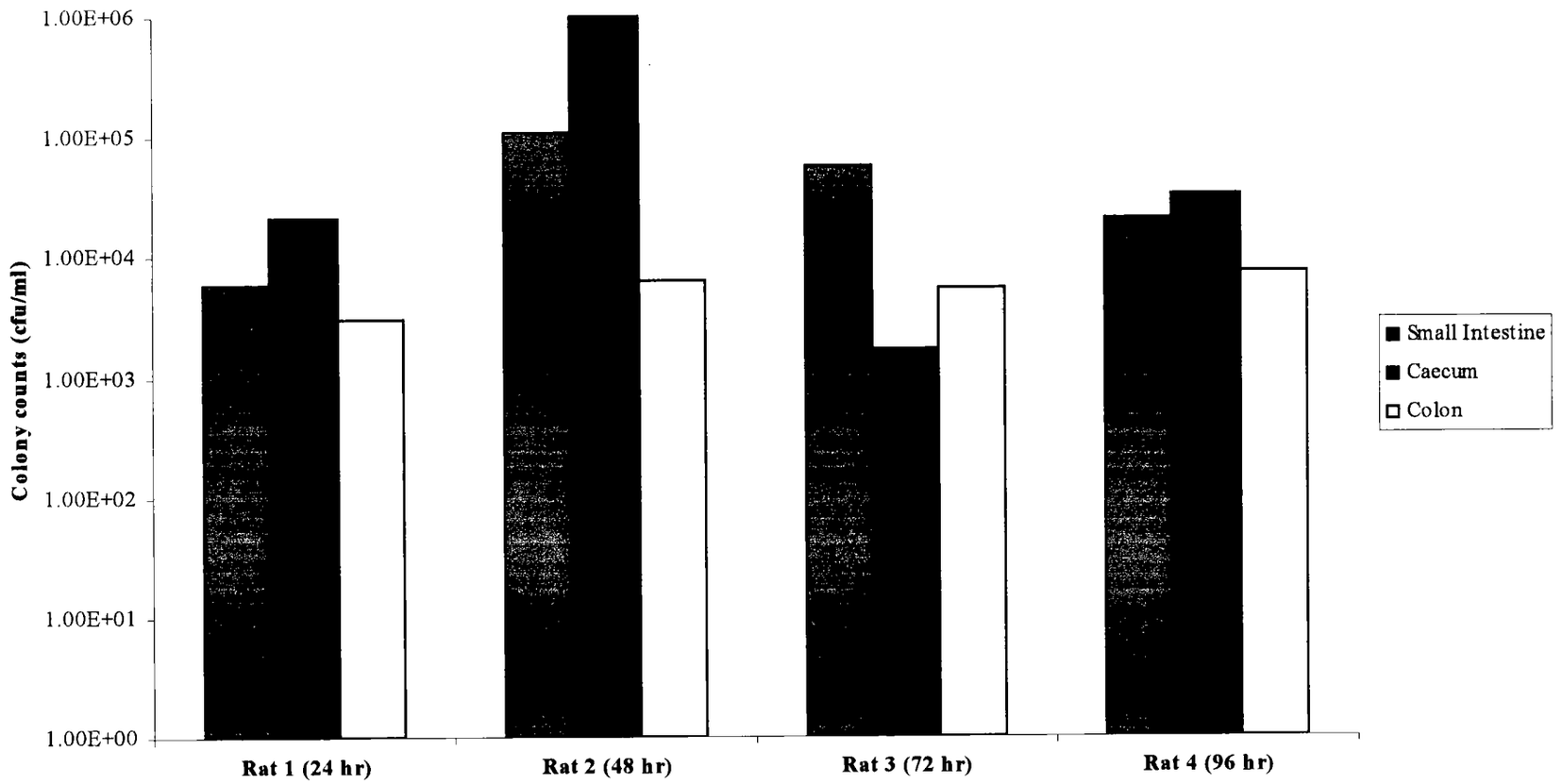
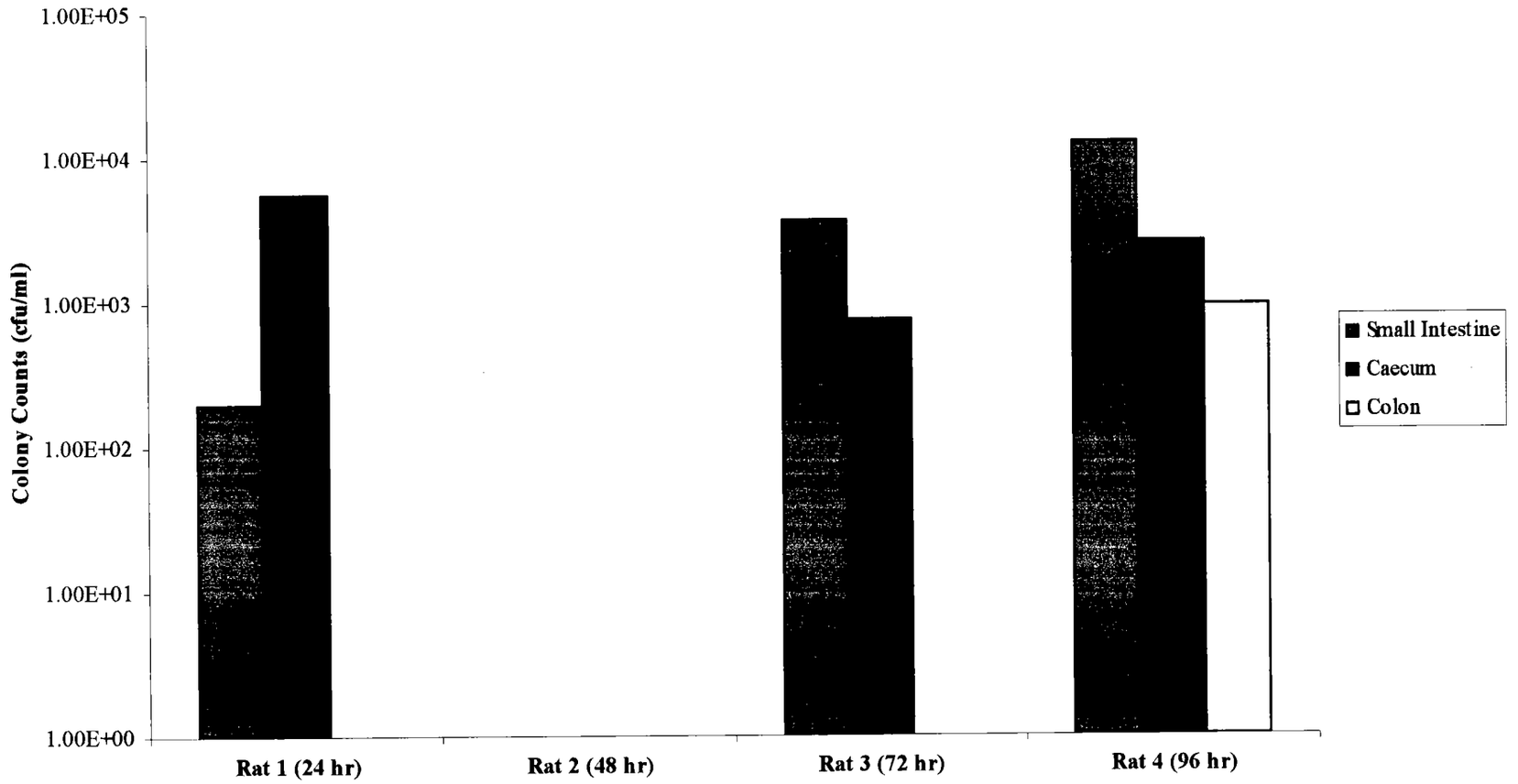


Figure 20: Distribution of Antibiotic Resistant Bacteria (10 μg/ml Cm) in Rat Gut Compartments of Rats inoculated with pKPSPgfpCm^R



gene. Faint PCR products of ~700 bp were visualised for Rat2 and Rat3 on day 3 (data not shown). However, these products were not sufficiently concentrated, even on re-amplification, to be sequenced.

Thus, from this set of experiments we were unable to demonstrate gene transfer by transformation in the rat gut using pKPSPsgfpCm^R as a model transmissible plasmid able to replicate both in Gram positive bacteria and *E. coli*. However, as there is a background of resistance it is impossible to conclude unequivocally that transformation is not a mechanism of gene transfer operating in gut ecosystems.

Transformation of the commensal human gut microflora

Transformation of the resident microflora was attempted with samples of fresh human saliva, 10% (w/v) human faecal slurry and a sample withdrawn from the colon fermentor. Transformation in all cases was attempted by adding 10μg pKPSPsgfpChl^R and pVACMC1 to separate samples and incubating the samples for 2 hours at 37°C. 200 μl aliquots were then plated on selective agar containing antibiotic concentrations known to allow transformation selection *in vitro*, but higher than normally used to eliminate as much background antibiotic resistance as possible (BHI + 50 μg/ml erythromycin and carboxymethylcellulose for pVACMC1 and BHI + 50 μg/ml erythromycin and 25 μg/ml chloramphenicol for pKPSPsgfpChl^R). There was still a high background of antibiotic resistance when 50μg/ml erythromycin was used for selection, but none of the colonies demonstrated endoglucanase activity. No colonies at all were detected on the plates containing 50 μg/ml erythromycin and 25 μg/ml chloramphenicol. These results indicate that no transformation of the resident microflora could be detected under the experimental conditions employed.

Detection of gene transfer to resident bacteria in gut simulations using the HSVTK system in conjunction with gfp markers

This section of work was carried out in collaboration with the groups of Prof Harry Gilbert (University of Newcastle) and Prof Mike Gasson (IFR, Norwich). The primary aim was to devise a system that would allow us to eliminate a donor strain in a gut simulation to facilitate detection of gene transfer events, making use of our expertise with *gfp* (18;19), gut fermentor systems (19) and FACS analysis. FACS

analysis of *Enterococcus faecalis* expressing the *gfp* gene was carried out. *E. faecalis* expressing *gfp* and a non-fluorescent strain of the same bacteria were mixed in equal numbers and sorted (Figure 21). There is considerable overlap between fluorescent and non-fluorescent *E. faecalis* meaning that to sort only fluorescent bacteria would result in the harvesting of very low bacterial numbers. As the numbers would be so low this was considered to be an unsuitable method for the detection of gene transfer. Two systems were tried making use of selective killing of a *Lactococcus* spp. donor in a gut simulation. The first made use of the bacteriocin lactococcin which selectively kills lactococci (IFR, Norwich) and the other system made use of the HSVTK system (University of Newcastle). Although lactococcin added to a cell lawn produced a clear zone due to cell lysis, it was not effective at inhibiting the growth of *L. lactis* during broth or plate culturing and thus was not a suitable killing method. The HSVTK system was introduced successfully into *E. coli* (IFR), but this strain only became available at the end of the project and no additional selection (*gfp* or Cm^R) was included. In the time remaining it was agreed that this section of the work should not be pursued further

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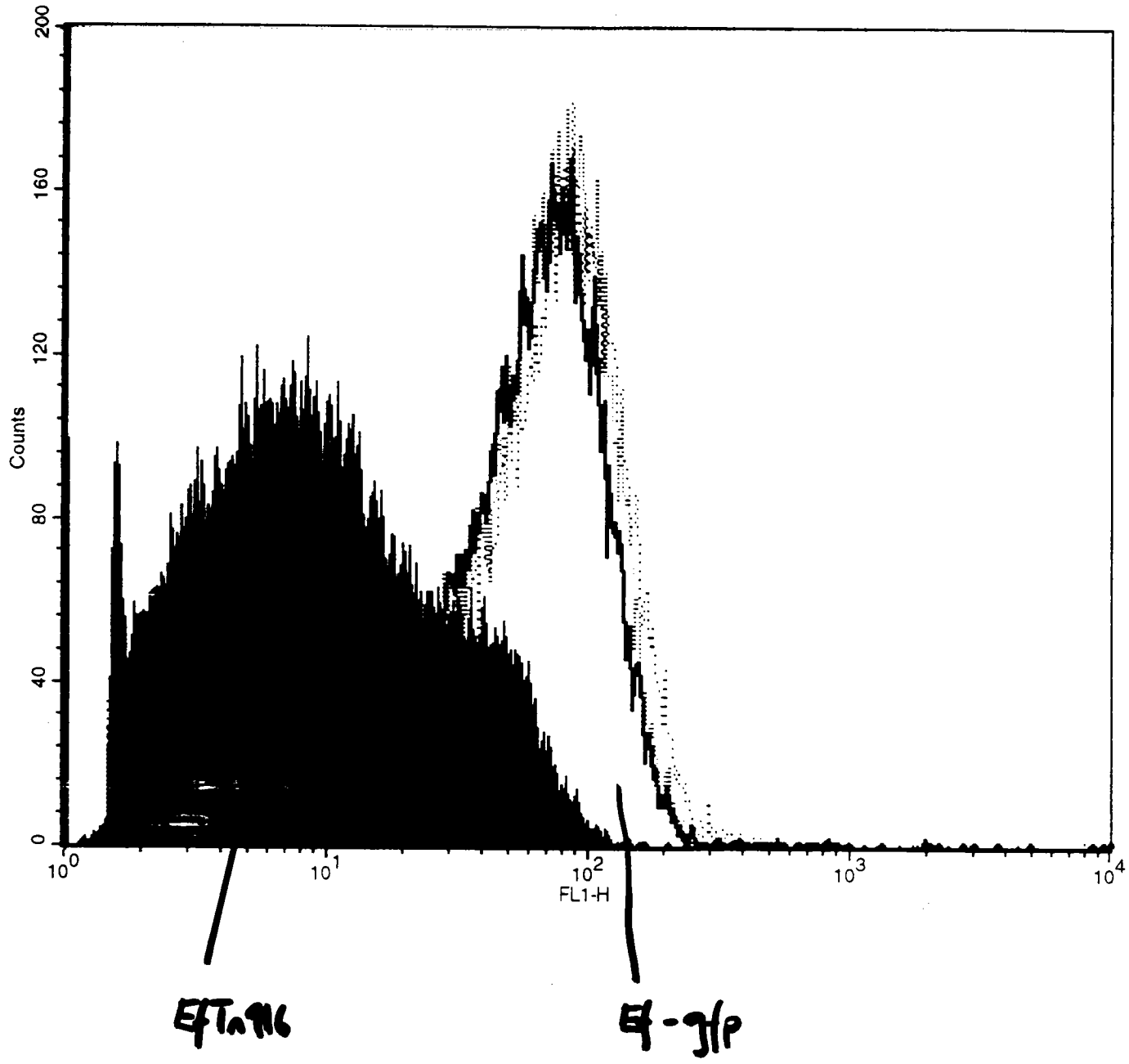

Figure 21. **FACS analysis of a 1:1 mixed population of *E. faecalis* Tn*916*. One fraction is fluorescent due to expression of the green fluorescent protein, whereas the other fraction is non-fluorescent**

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