Available online at www.sciencedirect.com
SCIENCE DIRECT
International Journal of Pharmaceutics 313 (2006) 29–35
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

Delivery of TEM β-lactamase by gene-transformed *Lactococcus lactis* subsp. *lactis* through cervical cell monolayer

Gagan Kaushal [a], Louis Trombetta [b], Raymond S. Ochs [b], Jun Shao [a,*]

[a] *Biotechnology and Drug Delivery Laboratory, Department of Pharmacy and Administrative Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Jamaica, NY-11439, USA*
[b] *Department of Pharmaceutical Sciences, College of Pharmacy and Allied Health Professions, St. John's University, 8000 Utopia Parkway, Jamaica, NY-11439, USA*

Received 13 October 2005; received in revised form 3 January 2006; accepted 12 January 2006
Available online 20 February 2006

Abstract

Lactococcus lactis subsp. *lactis* transformed with Plasmid ss80 (encoding the production and secretion of TEM β-lactamase) was used for the delivery of β-lactamase through the C-33A (cervix cell) monolayer. The viability of the cell monolayers co-cultured with *L. lactis* was examined by the trypan blue exclusion method. The integrity of the monolayers was monitored by measuring the transport of mannitol and propranolol as well as the transepithelial electrical resistance. The transport rate of β-lactamase through C-33A monolayer was increased by four- and nine-folds ($p < 0.05$) at the first hour by the transformed *L. lactis* compared to the free solution with or without presence of the untransformed *L. lactis*, respectively. This increase was gradually diminished after the 1st hour: it became 30 and 50% ($p < 0.05$) at 10 h. The presence of the untransformed *L. lactis* with free solution delivery also increased the transport rate by 100% at 1 h ($p < 0.05$) and 15% at 10 h ($p > 0.05$). The increase in transport rate by the transformed *L. lactis* is most probably due to the concentrate of β-lactamase on C-33A monolayer. When co-cultured with the *L. lactis*, the C-33A cell viability and the monolayer TEER remained steady for 10 h. The presence of *L. lactis* did not change the transport of propranolol and mannitol through the monolayers. In conclusion, the transformed *L. lactis* significantly ($p < 0.05$) increased the transport of β-lactamase through the cervical monolayers, indicating probiotic bacteria delivery may be a promising approach for protein delivery through the vagina.

Keywords: C-33A; *Lactococcus lactis*; TEM β-lactamase; Protein delivery; Normal flora; Bioavailability; Vaginal delivery

1. Introduction

Proteins are generally water soluble and cannot be delivered by non-invasive routes; they are usually delivered by injection. The frequent injection of these drugs causes pain, and inconvenience for the patients. There is a need for a non-invasive delivery system for these protein drugs. In the past two decades there have been enormous efforts in developing a non-invasive delivery system capable of achieving the required systemic blood levels of these protein drugs. However, not much success has been achieved mainly because of the poor absorption and extensive degradation of the protein drugs. To overcome these problems encountered in non-invasive protein drug delivery, one approach might be to utilize the genetically engineered normal flora as the delivery system.

Normal flora consists of the non-pathogenic bacteria that exist in the open tracts of the human body such as intestine, nostril, and vagina. By recombinant DNA technology, the normal flora can be genetically engineered to synthesize and secrete protein drugs. The normal flora has a natural tendency to adhere tightly to the epithelial cell surface of the channels where they normally reside (Tuomola and Salminen, 1998). This adherence provides an advantage for the recombinant bacteria in protein drugs delivery, since the bacteria will directly deliver the protein drugs onto the epithelial cell surface where the absorption will take place. The direct delivery of the protein drugs onto the epithelial surface will minimize the enzymatic and bacterial degradation of the protein drugs, and will also concentrate

Abbreviations: ATCC, American Type Culture Collection; AUC, area under the curve; C-33A, human cervix carcinoma; CPM, counts per minute; cfu, colony forming units; DMEM, Dulbecco's minimal essential media; FBS, fetal bovine serum; *L. lactis*, *Lactococcus lactis*; PBS, phosphate buffer saline; s-DMEM, supplemented DMEM; TEER, transepithelial electrical resistance

* Corresponding author. Tel.: +1 718 990 2510; fax: +1 718 990 6316.
E-mail address: shaoj@stjohns.edu (J. Shao).

0378-5173/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2006.01.013

the protein drugs at the absorption surface. Thus, the absorption may be increased. Studies have shown that fibrillae, strands of non-structured material associated with the lactobacillus cell surface, as well as surface adherent molecules enable the bacteria to attach to epithelial cells and other substrata (Tannock, 1992).

Lactic acid bacteria (LAB) are remarkably diverse group of gram-positive bacilli that are ubiquitous components of the normal indigenous flora of humans and animals. LAB have been isolated from the open tracts of the human body such as intestine, nostril, and vagina. LAB bacteria are often used in food processing and food preservation (Pouwels et al., 1998). Administration of viable lactobacillus strains has been described as therapeutic for diarrhea and other intestinal disorders (Sullivan and Nord, 2002), vaginitis (Reid et al., 1990), and urinary tract infections (Velraeds et al., 1996).

Lactococcus lactis, one of the safest LAB (Salminen et al., 1998), was chosen in the present study as a vector for the delivery of β-lactamase, a model protein of 29 kDa, through a model vaginal epithelial monolayer. The expression–secretion cassette (Plasmid ss80) for the host *L. lactis* was made and confirmed for expression and secretion of β-lactamase into the culture medium (Sibakov et al., 1991). Our previous studies showed that the *L. lactis* significantly increased the transportation of β-lactamase through Caco-2 monolayer and almost doubled the transportation rate as compared to solution form (Shao and Kaushal, 2004). Due to the intensive hepatic metabolism (1st-pass effect), most of the drugs show poor oral bioavailability even though the intestinal absorption is good. The vaginal route might be advantageous over the oral route since the former avoids the hepatic 1st-pass effect, and the vaginal epithelium is permeable to a wide range of molecules, like hormones, antimycotics, peptides and proteins, and its large surface area and rich blood supply make it a promising site for drug delivery (Richardson and Illum, 1992). In addition, a prolonged contact of a delivery system with the vaginal mucosa may be achieved more easily than at other absorption sites like the rectal or intestinal mucosa. In post-menopausal women, the reduced epithelial thickness may increase the drug absorption (Furuhjelm et al., 1980). Therefore, following our previous studies on normal flora delivery through Caco-2 monolayers, we have investigated the delivery of β-lactamase by normal flora through a model cervix epithelium—the C-33A monolayer in the current study.

The cervix is the channel between the vagina and uterus. Different types of cervix cell lines like C-33A, HeLa, CaSki, and many more have been used in numerous studies, most of which were cancer-oriented studies. C-33A is derived from the humans and shows epithelial morphology. It is one of the most commonly used cervical cell line and thus has been used for this study also. Drug absorption from these model cervical epithelia may be used to predict the in vivo absorption through vagina.

The main objective of the present study is to investigate the protein delivery efficacy by normal flora through C-33A monolayer in order to estimate the in vivo vagina absorption with β-lactamase as the model protein and *L. lactis* as the model normal flora. The secondary objectives of the present study are to explore the feasibility of using the in vitro cell culture technique to study the vaginal drug delivery by normal flora, and to examine the possible effects of the *L. lactis* on the C-33A monolayer.

2. Materials and methods

2.1. Materials

C-33A, a human cervical cell line, was purchased from ATCC (Rockville; MD, USA). *Lactococcus lactis* subsp. *lactis* (*L. lactis*), transformed with Plasmid ss80 containing the gene of β-lactamase was generously provided by Dr. Soile Tynkleynen (Valio Ltd., Helsinki, Finland). Dulbecco's modified eagle medium (DMEM), 0.25% trypsin with 0.2 g/l EDTA, fetal bovine serum (FBS), sodium pyruvate (11 mg/ml) and non-essential amino acids (100×) were obtained from Hyclone (Logan, UT, USA); β-lactamase and other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA); Transwell® inserts, tissue culture treated (0.4 μm pore size, 4.7 cm^2 surface area), and culture flasks were purchased from Costar Corporation (Cambridge, MA, USA); Bacto M17 broth and Bacto agar were purchased from Becton Dickinson (Sparks, MD, USA). D-mannitol-[1-^{3}H(N)] and DL-propranolol-[4-^{3}H] hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Culture of the C-33A cells

The C-33A cells were cultured in flasks in s-DMEM, DMEM supplemented with 10% (v/v) FBS, 100 I.U./ml penicillin–100 μg/ml streptomycin, sodium pyruvate (0.11 mg/ml), and 1% (v/v) non-essential amino acids at 37 °C in a 5% CO_2–95% air with high humidity. Upon confluence, the cells were harvested by the treatment of 0.25% trypsin with 0.2 g/l EDTA, resuspended in s-DMEM, seeded onto the polycarbonate filters of the transwells at a density of 3×10^4 cells/cm^2 and further incubated under normal cell culture conditions. The growth media was replaced every other day and transepithelial electrical resistance (TEER) was monitored periodically by a Millicell® ERS meter (Millipore, Bedford, MA) connected to a pair of Ag/AgCl electrodes. The monolayer became confluent and ready for transport studies after about 5 days when the TEER reading reached a plateau.

2.3. Electron microscopy

Electron microscopy was used to examine the adherence of *L. lactis* to the C-33A monolayer. The C-33A cells were seeded onto a glass cover slip (13 mm in diameter) in a six-well plate at a concentration of 3×10^4 cells/ml and were allowed to grow to form monolayer. The monolayer was then washed twice with phosphate buffered saline (PBS), and added with 2 ml of the *L. lactis* in antibiotic-free and FBS-free s-DMEM (4×10^7 cfu/ml). The plate was incubated at normal cell culture conditions for 3 h. The monolayer was washed five times with PBS and then fixed with 2.5% glutaraldehyde in PBS for 1 h at room temperature. After being washed five times with PBS, the monolayer was fixed for 30 min with 2% OsO_4, washed five times with PBS

and dehydrated in a graded series (30, 50, 70, 80, 90, and 100%) of acetone. The monolayer was dried in a critical-point dryer (Polaron E3000), sputter coated with the platinum in a Polaron E 5100 coater under an argon atmosphere at room temperature for 90 s, and examined under a Hitachi scanning electron microscope.

2.4. Effect of the L. lactis on viability of the C-33A monolayer

After the C-33A cells formed confluent monolayers in the transwells, the culture media was removed and the cells were washed twice with PBS. Then 2.6 ml of s-DMEM without antibiotics was added to the basal side. Freshly made *L. lactis* (either transformed or untransformed) suspension (1.5 ml) in antibiotic-free s-DMEM was added to the apical side ($\sim 4 \times 10^7$ cfu/well). The plate was centrifuged at $45 \times g$ for 5 min and was placed inside the incubation chamber for a certain period of time. The culture media was removed from the transwells. The C-33A cell monolayers in the transwells were rinsed twice with PBS solution and then treated with 500 μl of 0.25% trypsin with 0.2 g/l EDTA for 5–10 min to dislocate the C-33A cells from the bottom of the transwells. To each well 500 μl of s-DMEM was added to make a cell suspension, and 50 μl of the cell suspension was diluted with equal volume of 0.4% trypan blue dye. The viability of the C-33A cells was then counted by a Neubauer chamber under the microscope. The C-33A cell viability was determined right before the addition of the *L. lactis* (0 h) and at 6, 8, and 10 h after the addition. This study was repeated thrice in total of nine wells for each time point.

2.5. Effect of the L. lactis on the integrity of the C-33A monolayer

Once the C-33A cells formed confluent monolayers in the transwells, the monolayers were washed twice with PBS. Then 2.6 ml of antibiotic-free s-DMEM was added into the basal side. To the apical side, 1.5 ml of the freshly made *L. lactis* suspension in antibiotic-free s-DMEM containing either 35.5 nM D-mannitol-[1-^{3}H(N)] or 20 nM DL-propranolol-[4-^{3}H] hydrochloride was added ($\sim 4 \times 10^7$ *L. lactis* cfu/well). The cells were then further cultured. At each time interval, 100 μl of the media was taken from the basal side and replaced with equal amount of fresh media. The samples were withdrawn for 10 h. At the end of this experiment, 100 μl of the media was also taken from the apical side. The samples were placed in the scintillation vials, to which 10 ml of scintillation cocktail was added. These samples were counted for total counts per minute (cpm) by a Packard Tricarb LSC. TEER was also monitored during the study. This study was repeated on 3 different days.

2.6. Transport of β-lactamase through the C-33A monolayer

After the C-33A monolayer became confluent in the transwells, the basal media was replaced by 2.6 ml of the fresh s-DMEM without antibiotics in all six wells of each plate. The apical media was replaced by either 1.5 ml of freshly made *L. lactis* suspension in antibiotic-free s-DMEM ($\sim 4 \times 10^7$ *L. lactis* cfu/well), or 1.5 ml antibiotic-free s-DMEM containing 1000 mU β-lactamase with or without $\sim 4 \times 10^7$ untransformed *L. lactis*. The plate was centrifuged at $45 \times g$ for 5 min and cultured under normal cell culture conditions. At the pre-determined time periods, 50 and 200 μl samples were taken from the apical and the basal sides, respectively, and replaced by equal volume of the fresh s-DMEM without antibiotics. The samples were withdrawn upto 10 h. The assay of β-lactamase in these samples was carried out by the fluorimetry reported previously (Shao and Kaushal, 2004). Pure cell culture media was used as the blank control. The culture supernatant from the C-33A culture without the addition of the *L. lactis* or β-lactamase was also tested for any β-lactamase-like activity, and was found to be negative.

The AUC of the β-lactamase total amount–time curve at the apical side was calculated by the trapezoid method to determine the total β-lactamase exposure to the monolayer at the apical side over the experimental period of interest. The transport of β-lactamase was normalized by this AUC according to the following formula:

$$\text{Normalized transport rate} = X/\text{AUC}$$

where X is the cumulative amount of β-lactamase found in the basal side during the period of interest.

TEER was monitored throughout the absorption studies. A sample of the basal media was withdrawn at the end of this study to check the presence of bacteria in the basal sample. C-33A cell viability was also analyzed at the end of the study. Each transport study was done in three plates on 3 different days.

2.7. Data analysis

Student's *t*-test was used to compare the difference between the data of interest. Wherever possible, the data is presented as mean ± standard deviation.

3. Results and discussion

3.1. Electron microscopy

Electron microscopy was used to study the adherence of *L. lactis* to the cell monolayer. The scanning electron micrographs (Fig. 1) clearly show the binding of the *L. lactis* to the C-33A monolayer cell surface. Bacterial cell clusters can also be seen at the mucosal surface, as the *Lactococcus* species grow in groups or short chains. The results demonstrate the ability of the *L. lactis* to adhere to the cervical epithelial cells. Such adhesion is the key property that is utilized by the current protein delivery system to enhance the absorption. We hypothesize that through such adhesion the *L. lactis* will secrete β-lactamase directly onto the absorption surface to increase the bioavailability.

3.2. Effect of the L. lactis on the C-33A monolayers

The C-33A cell viability of the monolayer was examined at 0, 6, 8, and 10 h after being co-cultured with the *L. lactis* (either

Fig. 1. Scanning electron micrographs of the C-33A monolayer with the adhering *L. lactis*.

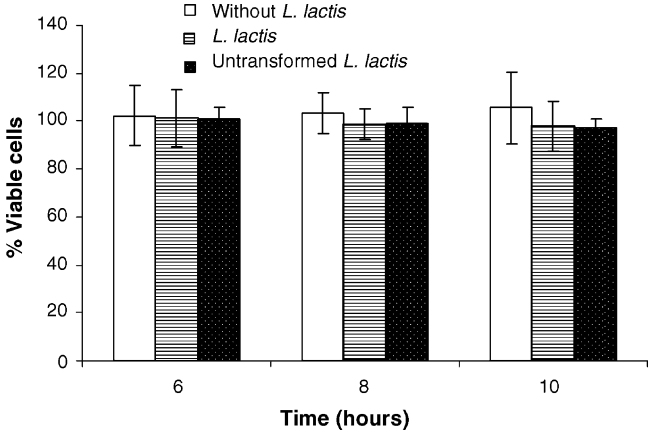

Fig. 2. C-33A cell viability when co-cultured with the *L. lactis*.

transformed or untransformed). It can be seen from Fig. 2 that the viability of the C-33A cells was maintained. Even at 10 h, the viability was still 98%.

The potential effect of *L. lactis* on the C-33A monolayer integrity during the transport studies was examined by both the paracellular marker D-mannitol-[1-^{3}H(N)] and the transcellular marker DL-propranolol-[4-^{3}H] hydrochloride as well as TEER measurement. Fig. 3 shows the transport of mannitol through the monolayers with or without the presence of the *L. lactis*. There was no statistical difference in the 10 h transported amount of mannitol between the two groups ($p > 0.05$). Mannitol has log *P* (partition coefficient) value and molecular weight as 2.50 and 182.2, respectively. It usually undergoes passive paracellular transport and thus is often used as a paracellular transport marker. The present results indicate that *L. lactis* did not significantly open the junctions between the C-33A cells in terms of mannitol permeation.

Propranolol has log *P* and molecular weight values as 2.53 and 259.3, respectively, and usually undergoes passive transcellular transport. Therefore, it has been used as a transcellular transport marker. As shown in Fig. 4, the amount of propranolol appearing at the basal side of the transwell is slightly more in case of the absence of the *L. lactis* (control). The LAB class of bacteria, which *L. lactis* belongs to, has a natural tendency to adhere to the intestinal mucosa (Tuomola et al., 1999). We think due to this adhesion of *L. lactis* to the C-33A monolayer, the passage of propranolol is slightly reduced. The results also indicate that the viability of the cells was not decreasing to a considerable extent in the presence of the *L. lactis*, otherwise the transport of propranolol would have increased substantially.

At the end of the transport study, both apical and basal media were sampled and analyzed to calculate the total radioactivity.

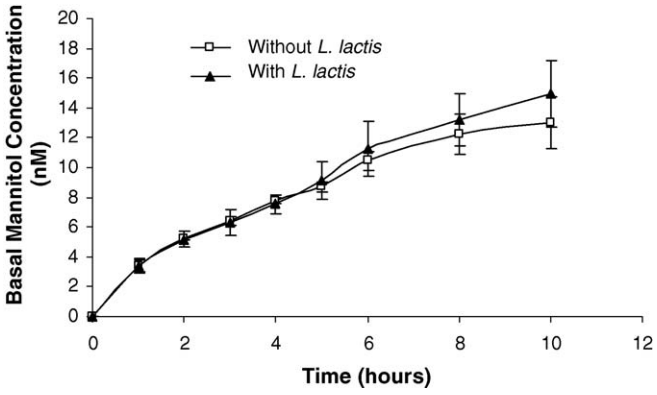

Fig. 3. Effect of the *L. lactis* on the transport of mannitol through the C-33A monolayer (mean ± S.D., $n = 9$).

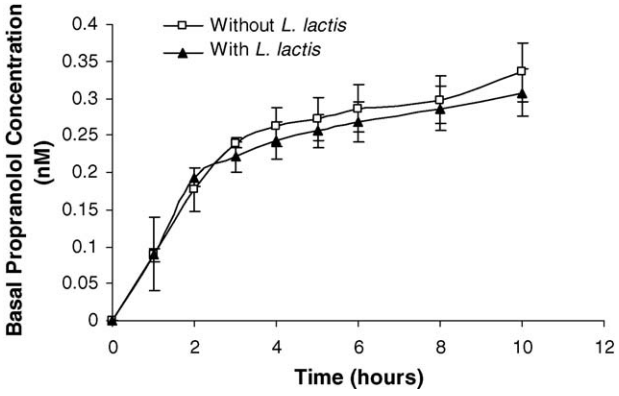

Fig. 4. Effect of the *L. lactis* on the transport of propranolol through the C-33A monolayer (mean ± S.D., $n = 9$).

Table 1
Recovery of mannitol and propranolol in the transwells

	Mannitol		Propranolol	
	No *L. lactis*	*L. lactis* added	No *L. lactis*	*L. lactis* added
With C-33A monolayer	97.2 ± 2.0	96.7 ± 3.2	92.5 ± 3.5	91.0 ± 3.3
No C-33A monolayer	101.2 ± 1.9	98.8 ± 1.5	96.8 ± 2.4	93.3 ± 1.8

The recovery is expressed as the percentage of the total radioactivity in the apical and basal chambers at the end of the study compared to the total radioactivity added at the beginning (mean ± S.D., $n = 9$).

The results (Table 1) demonstrate almost the full recovery of the radioactivity, indicating that the assay method is robust.

The TEER value of the monolayer was also monitored throughout all the studies with the cell monolayer in the transwells (Fig. 5). The TEER value remained stable during the 10 h studies, supporting the conclusion that the monolayer integrity was not compromised. It is well known that TEER can indicate the leakiness of the monolayer (the opening of the tight junction). However, TEER cannot sensitively detect the monolayer cell death (Mukherjee et al., 2004), which means that the effect of the *L. lactis* on the C-33A monolayer cannot be confirmed just by the TEER measurement. Thus, in addition to the TEER method, we have used mannitol and propranolol to examine the monolayer integrity and trypan blue assay to check the cell viability.

3.3. Transport of β-lactamase through the C-33A monolayers

Fig. 6 shows the absorption of β-lactamase through C-33A monolayer when delivered by the transformed *L. lactis*, or β-lactamase free solution with or without the untransformed *L. lactis*, which resulted in a 10 h accumulated transport rate of $3.0 \pm 0.08 \times 10^{-2}\,h^{-1}$, $2.3 \pm 0.09 \times 10^{-2}\,h^{-1}$, and $2.0 \pm 0.09 \times 10^{-2}\,h^{-1}$, respectively. These data shown above are normalized to the total drug exposure to the monolayers, which is the AUC of the β-lactamase amount–time curve in the apical side.

The untransformed *L. lactis* increased the 10 h transport rate of free solution by 15% ($p > 0.05$), while the transformed *L. lactis* delivery increased the transport rate by 50% ($p < 0.05$) compared to that by the free solution form. There was significant increase (30%, $p < 0.05$) in the transport of β-lactamase when delivered by the transformed *L. lactis* as compared to the untransformed *L. lactis* delivery, demonstrating the advantage of the transformed *L. lactis* over the untransformed *L. lactis*. This increase is most probably due to concentrating β-lactamase on the monolayer (absorption) surface by the transformed *L. lactis*.

Fig. 6 also shows that the amount of the transported β-lactamase in the basal side increased steadily over the 10 h period when delivered by the free solution form without the presence of the untransformed *L. lactis*. However, in both the other two cases, the transport rate appeared to be maximum at the first 2 h, and then decreased and became constant over the rest of the study period. Compared with the free solution alone, the transformed and the untransformed *L. lactis* increased the transport rate by five- and one-fold at 2 h, respectively (Table 2). But this increasing effect significantly reduced over the 10 h period, indicating the *L. lactis* exerted more significant effect at the beginning. Since the untransformed *L. lactis* was able to significantly ($p < 0.05$) increase the transport rate within the first 2 h, it is proposed that the untransformed *L. lactis* initially opened the intercellular junction of the C-33A cells. However, since this increase reduced and became insignificant ($p > 0.05$) over the 10 h period, it is proposed that the initial opening of the intercellular junction was almost completely closed later due to the reaction of the C-33A cells to the presence of the untransformed *L. lactis* through some unknown mechanisms.

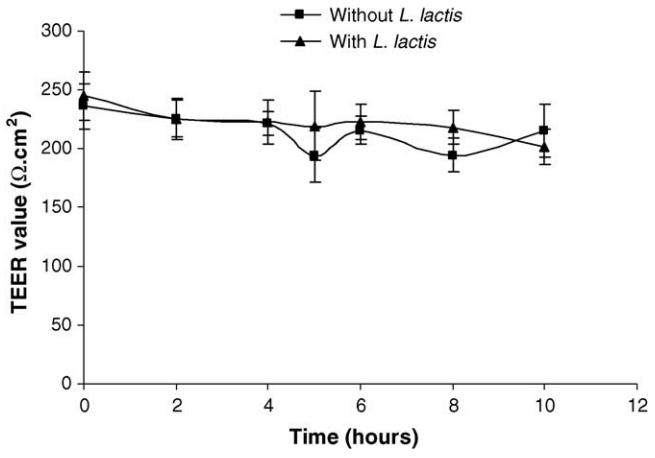

Fig. 5. TEER of the C-33A cell monolayer in the transwells during the β-lactamase transport study (mean ± S.D., $n = 9$).

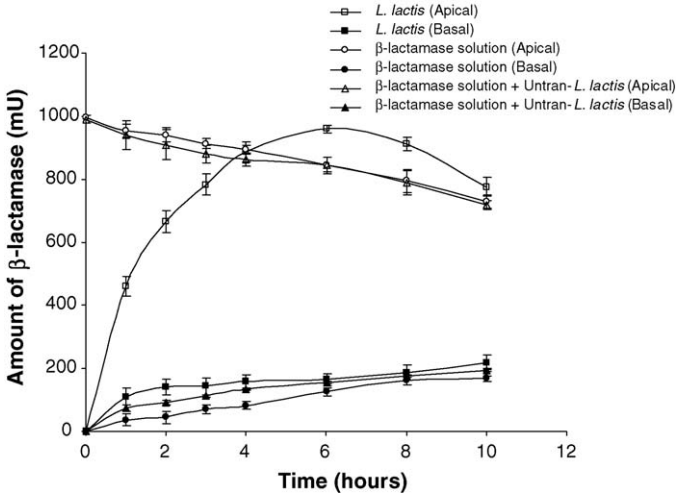

Fig. 6. Effect of the *L. lactis* on transport of β-lactamase through the C-33A monolayer (mean ± S.D., $n = 9$).

Table 2
Normalized transport rate of β-lactamase through C-33A monolayers ($\times 10^{-2}$ h^{-1}) (mean ± S.D., $n = 9$)

Time period (h)	β-lactamase solution	β-lactamase solution + untransformed *L. lactis*	Transformed *L. lactis*
1	3.8 ± 1.1	7.7 ± 1.9*	39.1 ± 4.4*,**
2	2.2 ± 0.09	4.8 ± 1.8*	13.6 ± 2.8*,**
10	2.0 ± 0.09	2.3 ± 0.09	3.0 ± 0.08*,**

* Significant difference from the rate by β-lactamase solution ($p < 0.05$).
** Significant difference from the rate by β-lactamase solution plus untransformed *L. lactis* ($p < 0.05$).

The similar phenomenon was observed with the transformed *L. lactis*. Therefore, both the untransformed and the transformed *L. lactis* might open the C-33A cellular junctions at beginning, and the opening was closed by the C-33A cells over a time period of about 2 h. Our previous studies showed that *L. lactis* could open the tight junctions of Caco-2 monolayer, and the opening was not closed during the 10 h transport study (Shao and Kaushal, 2004). Therefore, it can be concluded that the reactions to the *L. lactis* depends on the cell lines used.

Since the *L. lactis* is naturally present in the vaginal channel, it can be assumed that the interactions between the *L. lactis* and vaginal epithelium have already reached equilibrium in vivo. It is speculated that the addition of a certain amount of the *L. lactis* might disturb the equilibrium initially, and cause the opening of the junction of the vaginal epithelium to a certain limited degree, and then the vaginal epithelium may subsequently close the junction. As seen from the in vitro data, the increase in transport rate by this kind of interaction was not very significant. Such increase may be even less in vivo since cells in culture are more sensible to the environmental change than in vivo. Thus, it may be concluded that co-administration of untransformed *L. lactis* with β-lactamase to vagina will not lead to a significant increase of β-lactamase absorption except a possible slight initial increase. Also, the pre-existing flora does not have the effect similar to the initial impact on the epithelium that the newly administered *L. lactis* might have.

The initial opening of the intercellular junctions of the C-33A monolayer was not observed from the mannitol experiment. The reason may be the opening was limited and C-33A monolayer was already very permeable to mannitol but not to β-lactamase since mannitol is very small compared to β-lactamase. Therefore, such limited opening could not be reflected from the mannitol transport data.

Compared with the untransformed *L. lactis*, the transformed *L. lactis* significantly ($p < 0.05$) increased the β-lactamase transport rate by 407, 183 and 30% at 1, 2, and 10 h periods, respectively. These results clearly indicate that transformed *L. lactis* may facilitate β-lactamase transport by some mechanisms other than the opening of the intercellular junctions. It seems reasonable to state that the concentration of β-lactamase on the C-33A monolayer is the major factor for the absorption enhancement. As the transformed *L. lactis* adhered to the monolayer, it secreted β-lactamase directly onto the monolayer, resulting in a locally high concentration. Over the time, this localized β-lactamase would diffuse into the bulk culture media in the transwell, causing the decrease in the β-lactamase transport rate. This may explain why the increase in β-lactamase transport rate by the transformed *L. lactis* when compared with the untransformed *L. lactis* gradually reduced over the time.

As discussed before, co-administration of untransformed *L. lactis* would not significantly increase the β-lactamase absorption in vivo since the initial opening of intercellular junction by administration of *L. lactis* would be very limited, and even may not happen in vivo. However, the transformed *L. lactis* may still be able to significantly enhance the β-lactamase absorption in vivo. First, through the adherence to the vaginal epithelium, the transformed *L. lactis* will secrete β-lactamase onto the vaginal epithelium to concentrate the protein drug on the absorption surface and reduce the exposure of the protein drug to a hostile environment. Second, the transformed *L. lactis* will continuously produce and secrete β-lactamase, and due to its adhesive property it usually can stay in the vagina for a certain period before being eliminated, so that the transformed *L. lactis* can provide a prolonged delivery mechanism.

The results of the present study also demonstrated the absence of *L. lactis* in the basal sample as checked at the end of transport study, proving that it is the β-lactamase that was transported through the monolayers and rules out the possibility of *L. lactis* getting transported through the C-33A monolayer.

The current study shows that the *L. lactis* delivery resulted in a normalized β-lactamase transport rate of 3.0×10^{-2} h^{-1} through C-33A monolayer, which is 50% higher than the transport rate through Caco-2 monolayer, a model intestinal epithelium (Shao and Kaushal, 2004). This result is consistent with the previous findings that vagina is more permeable than intestine (Richardson and Illum, 1992). Although vagina may provide certain advantages for protein delivery, one should keep in mind that this route is limited only to females, and this route is not as convenient as the oral route.

The present study is just the initial step to explore the feasibility and efficacy of using normal flora to deliver protein drugs via vaginal route. Although the results show that the *L. lactis* can significantly increase the transport of β-lactamase, it is still very far away from clinical use. There are many questions to be answered, such as the in vivo delivery efficacy, how long the normal flora can stay, and the dose–response effect. In addition, even though this normal flora system may provide some advantages such as enhanced absorption and prolonged delivery, it may not be suitable for cases where very accurate dosage and/or short period of action are required or in other cases where immediate reactions are expected. In stead, it may have a wide application for drugs such as growth hormones in the treatment of dwarfism or other cases where a long-term sustained delivery is preferred.

4. Conclusions

The transport of β-lactamase through the C-33A monolayer was significantly increased by the transformed *L. lactis* compared to the free solution form with or without the untransformed *L. lactis*, indicating that the transformed *L. lactis* may be used as an efficient protein delivery vehicle through vaginal route.

L. lactis did not cause appreciable C-33A monolayer death or integrity disruption, demonstrating the feasibility of using the cell culture technique to evaluate the protein delivery via genetically engineered normal flora.

Acknowledgement

We would like to thank Dr. Soile Tynkleynen, Valio Ltd., R&D (Meijeritie 4, Helsinki, Finland) for generously providing the strain *Lactococcus lactis* subsp. *lactis* (pKTH1805).

References

Furuhjelm, M., Karlgren, C., Carlstrom, K., 1980. Intravaginal administration of conjugated estrogens in post-menopausal women. Int. J. Gynecol. Obstet. 7, 335–339.

Mukherjee, T., Squillante, E., Gillespie, M., Shao, J., 2004. Transepithelial electrical resistance is not a reliable measurement of the Caco-2 monolayer integrity in the transwell. Drug Deliv. 11, 11–18.

Pouwels, P.H., Leer, R.J., Shaw, M., den Bak-Glashouwer, M.J.H., Tielen, F.D., Smit, E., Martinez, B., Jore, J., Conway, P.L., 1998. Lactic acid bacteria as antigen delivery vehicles for oral immunization purposes. Int. J. Food Microbiol. 41, 155–167.

Reid, G., Bruce, A.W., McGroarty, J.G., Cheng, K.J., Costerton, J.W., 1990. Is there a role for lactobacilli in prevention of urogenital and intestinal infections? Clin. Microbiol. Rev. 3, 335–344.

Richardson, J.L., Illum, L., 1992. The vaginal route of peptide and protein drug delivery. Adv. Drug Deliv. Rev. 8, 341–366.

Salminen, S., von Wright, A., Morelli, L., Marteau, P., Brassart, D., de Vos, W.M., Fonden, R., Saxelin, M., Collins, K., Mogensen, G., Birkeland, S.E., Mattila-Sandholm, T., 1998. Demonstration of safety of probiotics—a review. Int. J. Food Microbiol. 44, 93–106.

Shao, J., Kaushal, G., 2004. Normal flora: living vehicles for non-invasive protein drug delivery. Int. J. Pharm. 286, 117–124.

Sibakov, M., Koivula, T., von Wright, A., Palva, I., 1991. Secretion of TEM β-lactamase with signal sequences isolated from the chromosome of *Lactococcus lactis* subsp. *lactis*. Appl. Environ. Microbiol. 57, 341–348.

Sullivan, A., Nord, C.E., 2002. The place of probiotics in human intestinal infections. Int. J. Antimicrob. Agents 20, 313–319.

Tannock, G.W., 1992. The lactic microflora of pigs, mice and rats. In: Wood, B.J.B. (Ed.), The Lactic Acid Bacteria in Health and Disease. Elsevier Applied Science, London and New York, pp. 21–48.

Tuomola, E.M., Salminen, S.J., 1998. Adhesion of some probiotic and dairy *Lactobacillus* strains to Caco-2 cell cultures. Int. J. Food Microbiol. 41, 45–51.

Tuomola, E.M., Ouwehand, A.C., Salminen, S.J., 1999. Human ileostomy glycoproteins as a model for small intestinal mucus to investigate adhesion of probiotics. Lett. Appl. Microbiol. 28, 159–163.

Velraeds, M.M., van der Mei, H.C., Reid, G., Busscher, H.J., 1996. Inhibition of initial adhesion of uropathogeinc *Enterococcus faecalis* by biosurfactants from *Lactobacillus* isolates. Appl. Environ. Microbiol. 62, 1958–1963.