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Oral delivery of β-lactamase by *Lactococcus lactis* subsp. *lactis* transformed with Plasmid ss80

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Abstract

The objective was to use normal flora to deliver protein/peptide drugs orally. A probiotic bacterium, *Lactococcus lactis* subsp. *lactis* (*L. lactis*) transformed with Plasmid ss80, which made it able to synthesize and secrete β-lactamase, a 29 kDa protein, was used as the delivery system for β-lactamase. Oral absorption of β-lactamase in rats when delivered by this *L. lactis* system was investigated. The oral bioavailability of β-lactamase delivered by 3×10^7 of the *L. lactis* was equivalent to 209 mU of i.v. dose, and the estimated relative bioavailability was 16.7%. When delivered by β-lactamase free solution form, the relative oral bioavailability was 4.7%, which increased to 6.0% when co-administered with 3×10^7 of the untransformed *L. lactis*. The results demonstrated that the *L. lactis* significantly increased the β-lactamase oral bioavailability by 2–3-folds ($p < 0.01$), the mean residence time (MRT) by 3–4 times ($p < 0.01$), and the mean absorption time (MAT) by 6–14 times ($p < 0.01$), as compared to the free solution form with/without the untransformed *L. lactis*. In conclusion, the *L. lactis* is more efficient in delivering β-lactamase orally compared with the free solution form. It also provides a sustained delivery mechanism for β-lactamase. Gene-transformed normal flora may be used as an efficient and sustained delivery system for protein drugs through oral route.

Keywords: *Lactococcus lactis*; β-Lactamase; Normal flora; Protein oral administration; Pharmacokinetics; Bioavailability; Sustained delivery

1. Introduction

Many proteins or polypeptides are now widely being used or tested as regulatory substances, enzyme inhibitors or antibodies to combat human diseases. Almost all the biological products are delivered by injections. Very few have been successfully formulated into products which are delivered through non-invasive routes. Injections, especially long-term use, are very inconvenient, painful, and unsafe. Non-invasive delivery methods are facing two common problems: (1) very low absorption and (2) extensive degradation before absorption takes place. The oral route has been extensively explored for protein drug delivery because the intestine is a naturally designed absorption organ. Many delivery techniques have been tried to enhance the oral absorption of protein drugs, such as protein structure modification (Clement et al., 2002; Kipnes et al., 2003), use of absorption enhancers (Uchiyama et al., 1999; Onuki et al., 2000; Thanou et al., 2000), enzyme inhibitors (Yamamoto et al., 1994; Agarwal et al., 2001) enteric coatings (Lee et al., 1999; Hosny et al., 2002), and nanoparticle delivery (Sakuma et al., 2002). There is still lack of an oral delivery system to achieve the required systemic blood levels of these protein drugs.

We have hypothesized that normal flora might be used to deliver protein drugs orally based upon the following rationales. By recombinant DNA technology, normal flora can be genetically engineered to produce and secrete protein drugs. Once placed into the tracts where they normally reside in vivo, this normal flora due to their natural bioadhesive properties (Salminen et al., 1998) can deliver the drugs directly onto the absorption mucosa. This will prevent the degradation of the protein drugs and concentrate them on the absorption surface. Therefore, the gene-transformed normal flora may be an effi-

Abbreviations: ATCC, American type culture collection; *L. lactis*, *Lactococcus lactis* subsp. *lactis*; O.D., optical density; PBS, phosphate buffer saline; cfu, colony forming units; AUC, area under the curve; AUMC, area under the first moment curve; MRT, mean residence time; MAT, mean absorption time; TCA, trichloroacetic acid

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cient delivery system for protein drugs and will also minimize the enzymatic and bacterial degradation of the protein drugs.

Lactococcus lactis subsp. *lactis* (*L. lactis*) is one of the safest strains in the lactobacillus family, is used in the present study. This strain has been transformed with Plasmid ss80 (Sibakov et al., 1991). Therefore it can synthesize and secrete β-lactamase, a 29 kDa protein (Christov et al., 2004), which is the model protein used in our study. Lactobacillus strains have been used as attractive candidates for expressing foreign antigens and mucosal immunization (Gilbert et al., 1996; Zegers et al., 1999; Shaw et al., 2000; Grangette et al., 2001; Scheppler et al., 2002, 2005). Geoffroy et al. (2000) have studied the fate of *L. lactis* in the digestive tract of mice and have found *L. lactis* to be transient bacterium in the digestive tract. Cross (2002) has also addressed that probiotic lactobacilli, following oral delivery, can survive in the human GI tract. In a study where human feeding of *L. lactis* was performed, a substantial portion of *L. lactis* was seen to survive the passage of the gastrointestinal tract within 3 days after consumption (Klijn et al., 1995). Therefore the gene-transformed *L. lactis* may provide a sustained delivery mechanism for β-lactamase.

In our previous study (Shao and Kaushal, 2004), it was shown that the *L. lactis* can significantly increase the transportation of β-lactamase through Caco-2 monolayer in vitro and almost double the transportation rate as compared to the solution form. The present study was carried out to test the concept of using normal flora as a protein drug delivery system in vivo, and to study the delivery efficiency by such a system. Therefore, we delivered the β-lactamase to the rats through oral route by this transformed *L. lactis*, monitored the plasma concentration after the dosing, and compared the bioavailability by the *L. lactis* with that by the free solution form with or without the co-administration of the untransformed *L. lactis*.

2. Materials and methods

2.1. Materials

L. lactis subsp. *lactis* (untransformed *L. lactis*, ATCC no. 11454) was purchased from ATCC (Rockville, MD, USA). *L. lactis* subsp. lactis, transformed with Plasmid ss80 (thereafter referred as *L. lactis*, unless otherwise specified) encoding β-lactamase and its secretion signal was generously provided by Dr. Soile Tynkleynen (Valio Ltd., Helsinki, Finland). BBD Dry Slide Nitrocefin, Bacto M17 broth and Bacto agar were purchased from Becton Dickinson (Sparks, MD). Ampicillin, β-lactamase (from *Bacillus cereus* EC 3.5.2.6), ascorbic acid, ethylenediamine tetraacetic acid (EDTA), trichloroacetic acid (TCA) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). All the chemicals were of analytical grade.

Female Sprague–Dawley rats weighing 300 ± 25 g, purchased from Taconic Farms, Germantown, NY, were used in these experiments. The rats were acclimated to their surrounding for at least 1 week and were housed in the same room. The microisolator cages were used with two animals in each cage. They were housed on a 12 h light/dark cycle and a relative humidity of 40–60%. Animals were restrained from food and water 12 h prior to the experiments. All the experiments were performed under a vertical Laminar Flow Hood.

2.2. Verification of β-lactamase production and secretion by L. lactis

This verification was first carried out by Nitrocefin test. The test is based on the production of color when the substrate, nitrocefin, is exposed to β-lactamase (O'Callaghan et al., 1972). By a Pasteur pipette, a small drop of distilled water was dispensed onto the reaction area of BBL Dry Slide Nitrocefin. Then the test sample was generously smeared onto the moistened reaction area of BBL Dry Slide Nitrocefin. The reaction area was examined for the appearance of a color change from yellow to pink in 1–50 min. The test samples were a 12-h grown culture of *L. lactis* suspension and a standard β-lactamase solution. The 12-h grown *L. lactis* suspension was also centrifuged at $9000 \times g$ for 5 min, and then both the supernatant and pellets were tested for β-lactamase activity as well.

The production and secretion of β-lactamase by *L. lactis* was further confirmed by SDS-PAGE assay (Sambrook et al., 1987). To four volumes of the supernatant solution from the 12 h culture of *L. lactis*, one volume of 8% TCA was added. This was incubated for 10 min at 4 °C. The tube was centrifuged at $17{,}530 \times g$ for 5 min. The supernatant was removed. The pellet was washed with 200 μl cold acetone and centrifuged at $17{,}530 \times g$ for 5 min. This washing step was repeated twice. The pellets were dried at 95 °C for 5–10 min to drive off the acetone. To four parts of this sample, one part of 5× SDS gel-loading buffer was added. This was boiled for 10 min at 95 °C before loading onto the polyacrylamide gel. Mini-PROTEAN 3 (Bio-rad) apparatus was used for running the gel according to the manufacturer's instructions. Samples (15 μl) were loaded into the bottom of the wells. The electrophoresis apparatus was attached to an electric supply and a voltage of 8 V/cm was applied to the gel. After the dye has moved into the resolving gel, the voltage was increased to 15 V/cm and the gel was run until the bromophenol blue reaches the bottom of the resolving gel. The gel was then fixed and stained with Coomassie Brilliant Blue.

2.3. L. lactis growth and secretion of β-lactamase in M17 broth in vitro

3×10^7 colony forming units (cfu) of *L. lactis* in growing phase were suspended in 3 ml of M17 media and incubated at 35 ± 2 °C. Triplicate samples of 200 μl were withdrawn from this tube at predetermined time intervals and replaced with equal amounts of fresh M17. The samples were assayed for *L. lactis* and β-lactamase.

L. lactis count was determined by performing viable count and measuring the optical density (O.D.) of the culture. Serial dilution of the *L. lactis* culture was prepared and each dilution was spread over the whole surface of the agar plate by a spreader. The plates were then placed in the incubator at 35 ± 2 °C for 24 h and the growing cfu were counted. The *L. lactis* concentration was calculated based on the counts and dilution factor.

The absorbance at 600 nm of the *L. lactis* suspension was also measured as the secondary method for *L. lactis* assay.

For β-lactamase quantitation, 0.2 ml of sample was mixed with 0.8 ml of 6.25 mM ampicillin at 25 °C. The reaction was allowed to last for exact 10 min and then 0.1 ml of 8% trichloroacetic acid at 4 °C was immediately added to cease the reaction. This solution was diluted to 5 ml with 0.5 M acetate buffer at pH 4 and then heated at 100 °C for 30 min. After cooling to room temperature, 0.1 ml of each sample was placed in 96 well plates and the fluorescence was read by a microplate reader at an excitation wavelength of 340 nm and an emission wavelength of 465 nm.

2.4. In vivo β-lactamase oral absorption

The research protocol was approved by Institutional Animal Care and Use Committee of the St. John's University and confirmed to the NIH guide for the use and care of laboratory animals. The animals were grouped randomly with six animals in each group and fasted overnight before dosing. Each animal was administered 0.2 ml of the dose through oral gavage or tail vein injection according to the dosing schedule given in Table 1. Blood samples of 0.5 ml were withdrawn from the tail at the predetermined time periods after the administration. All blood samples were collected in an Eppendorf tube to which 25 mg of EDTA (anticoagulant) had been added. Plasma was immediately separated from erythrocytes by centrifugation at 9000 × *g* for 3.5 min and 0.2 ml of plasma was collected in a separate Eppendorf tube. β-Lactamase in these plasma samples was analyzed by the HPLC method.

2.5. β-Lactamase HPLC assay

An HPLC method was used for the determination of β-lactamase concentration in the plasma. The samples were processed by the method described by Baker (1999) with a few modifications. To 0.2 ml of each of the plasma sample, 0.4 ml of 6.25 mM ampicillin (substrate) was added. The reaction mixture was incubated at 37 °C for 30 min and then 0.1 ml of 60% TCA at 4 °C was immediately added to cease the reaction. The solution was centrifuged at 9000 × *g* for 5 min and 0.5 ml of the supernatant was added to 2 ml solution of 0.5 M acetate buffer at pH of 5 containing ascorbic acid (0.5 mg/ml) and EDTA (50 mM). The resulting solution was heated at 100 °C for exactly 30 min and was allowed to cool down to the room temperature. The samples were then analyzed by the HPLC method.

Table 1
Experimental design for the in vivo study on β-lactamase absorption in rats (six rats/group)

Group	Dose	Delivery route
I	252 mU of β-lactamase in solution	i.v. injection
II	1008 mU of β-lactamase in solution	Oral gavage
III	1008 mU of β-lactamase in solution plus 3×10^7 cfu of the untransformed *L. lactis*	Oral gavage
IV	3×10^7 cfu of the *L. lactis* (ss80)	Oral gavage

The HPLC system consisted of a Waters 600E system controller, a Waters 717 Autosampler, and a Waters 470 Scanning fluorescence detector. The separation was done on a μ Bondapak C18 cartridge column (3.9 mm × 300 mm i.d.). The injection volume was 10 μl. The mobile phase was 80% of 0.1 M phosphate buffer (pH 5.0) and 20% of acetonitrile with a flow rate of 1.5 ml/min. The column effluents were monitored at excitation and emission wavelength of 410 and 475 nm, respectively, for a run time of 11 min, and the peak of interest was seen at the retention time of 9 min. Two standard curves were constructed by analysis of the peak area against the concentration of the β-lactamase in two different concentration ranges of 0.252–1.26 and 1.26–12.6 mU/ml, which were prepared by spiking the blank plasma with β-lactamase standard solution. The concentration of β-lactamase in the plasma samples was determined by the standard curve method.

2.6. Pharmacokinetic parameters and statistics

The PK parameters were calculated by the noncompartmental analysis (Gibaldi and Perrier, 1982). The maximum plasma concentration (C_{max}) and the time (T_{max}) when it was reached were observed from the β-lactamase plasma concentration–time profile. Statistical analysis was performed using Student's *t*-test with $\alpha = 0.05$ as the minimal level of significance.

3. Results

3.1. Verification of β-lactamase production and secretion by the L. lactis

The nitrocefin assay is the test of the presence of β-lactamase. After the sample is added to the Nitrocefin slide, only the sample containing β-lactamase can change the color of the reaction area from yellow to pink. In the present study, the test samples were the *L. lactis* culture suspension, the supernatant of the culture and the *L. lactis* cells. In all cases, the yellow color of the Nitrocefin slide changed to pink. The results demonstrate the presence and secretion of β-lactamase by the *L. lactis*.

The gel after the SDS-PAGE analysis shows a band from the *L. lactis* culture media corresponding to the standard β-lactamase (Fig. 1). The size of these bands (protein) matched with the molecular weight marker in the range of 25–37 kDa. Sibakov et al. (1991) reported the size of β-lactamase secreted

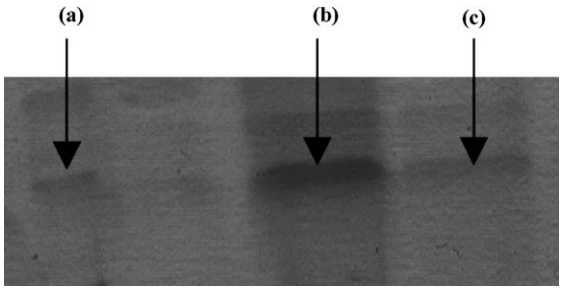

Fig. 1. Coomassie blue-stained gel after SDS-PAGE analysis. (a) Molecular weight marker, (b) *L. lactis* culture supernatant and (c) standard β-lactamase.

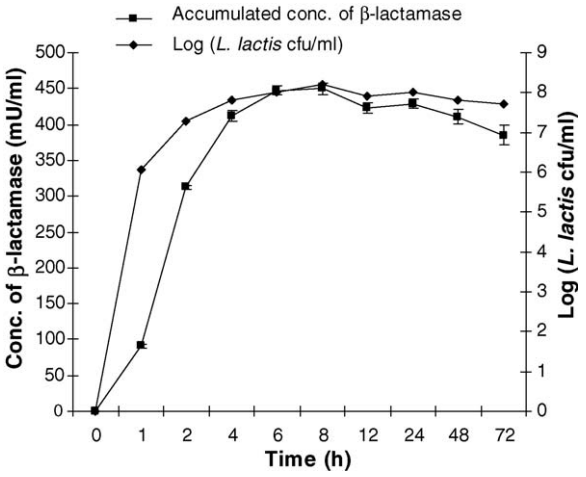

Fig. 2. Growth and secretion of β-lactamase from 3×10^7 cfu of *L. lactis* in 3 ml of M17. Three samples of the culture suspension were withdrawn at each predetermined time points and replaced by equal volume (600 μl) of the fresh M17 broth. β-Lactamase in the samples was analyzed by the fluorimetric assay.

from this *L. lactis* was about 30 kDa. Thus the present SDS-PAGE analysis further confirms the presence of β-lactamase in the culture media which was secreted by the *L. lactis*.

3.2. *L. lactis* growth and production of β-lactamase in M17 broth

This study was carried out to quantitate the maximum amount of β-lactamase secreted from 3×10^7 cfu of the *L. lactis* in 3 ml of M17. Fig. 2 shows the growth of the *L. lactis* and the accumulated concentration of β-lactamase. The data presents a typical bacterial growth curve: initial log-phase growth followed by a stationary phase and a decline phase because of the limited nutrition and space. The secretion of β-lactamase appeared correlated with the growth of the *L. lactis*. The cumulative amount of β-lactamase up to 24 and 72 h was 1250 and 1350 mU, respectively. The amount of β-lactamase in the culture media increased initially, then reached a plateau at 6–8 h and then decreased slightly. The decease was most probably caused by the proteases secreted by the *L. lactis* (Foucaud-Scheunemann and Poquet, 2003).

3.3. Oral absorption of β-lactamase in rats

Fig. 3 shows the plasma concentration profile of β-lactamase after oral administration to the rats. The pharmacokinetic parameters are listed in Table 2. When the β-lactamase free solution was given, the mean C_{max} was 1.5 mU/ml, T_{max} was between 3 and 5 h, and no β-lactamase was detected in the plasma at 24 h. The co-administration of the untransformed *L. lactis* with the β-lactamase free solution showed similar β-lactamase plasma concentration profile as the free solution alone, which indicates that the untransformed *L. lactis* did not change the absorption of β-lactamase. When the transformed *L. lactis* was administered, the mean C_{max} was 2.1 mU/ml (40% higher than the free solution case, $p < 0.05$), T_{max} was between 6 and 12 h, and at 72 h after the administration, β-lactamase was still detectable in the plasma. This delayed T_{max} (compared with the free solution

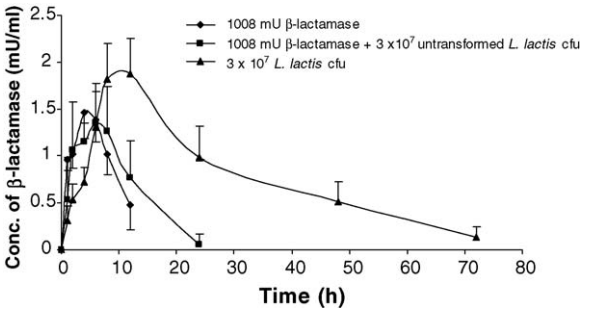

Fig. 3. β-Lactamase plasma concentration after the oral administration of various forms.

case) is probably because it took time for the *L. lactis* to grow and start to secrete β-lactamase in vivo, as seen from the in vitro study (Fig. 2), the maximum concentration occurred around 6 h.

Compared to the free solution form, the *L. lactis* increased the MRT by about three times ($p < 0.01$), and the MAT by 6–13 times ($p < 0.01$). Therefore, the *L. lactis* rendered a prolonged delivery mechanism, providing the advantage of much less frequency of drug administration. This result is within the expectation because the *L. lactis* possessed the ability to adhere to the intestine mucosa and gradually secrete β-lactamase for absorption. But this kind of adhesion is not permanent according to the β-lactamase plasma concentration profile. Since no β-lactamase was detected at 96 h after the administration of the *L. lactis*, it can be assumed that most of the *L. lactis* were eliminated (or about to be eliminated) out of the body by that time. This phenomenon is supported by a previous report (Geoffroy et al., 2000), which showed that the *L. lactis* was a non-colonizing organism and was transient in the digestive tract of mice. The non-permanent adhesion of the *L. lactis* is desired for the delivery of most of the drugs so that the treatment period can be easily adjusted and controlled. These results also suggest that *L. lactis* was able to sustain its activity in the digestive tract for a sufficient length of time for protein delivery.

According to the AUC after the i.v. administration of 252 mU of β-lactamase, the relative bioavailability from the oral dose of 1008 mU of β-lactamase in free solution was calculated to be 4.7%. When this oral dose co-administered with the untransformed *L. lactis*, there was a 27% increase in the bioavailability, but without any statistical significance ($p > 0.05$). The oral bioavailability of β-lactamase when delivered by 3×10^7 of the transformed *L. lactis* was equivalent to 209 mU of i.v. dose. Since there was no way to accurately measure the total amount

Table 2
Pharmacokinetic parameters of β-lactamase in rats after i.v./oral administration (mean ± S.D., $n = 6$)

Group	I	II	III	IV
AUC (mU h ml^{-1})	79.4 ± 12.9	14.9 ± 2.4	18.9 ± 5.5	65.7 ± 10.8*
Bioavailability (%)	100	4.7 ± 0.76	6.0 ± 1.8	16.7 ± 2.7*
C_{max} (mU/mL)	–	1.5 ± 0.06	1.7 ± 0.18	2.1 ± 0.27*
T_{max} (h)	–	3–5	4–6	6–12
MRT (h)	5.1 ± 0.9	6.3 ± 1.6	7.5 ± 2.2	22.9 ± 5.0*
MAT (h)	–	1.2 ± 1.6	2.4 ± 2.2	17.8 ± 5.0*

* Significant difference from groups II and III ($p < 0.05$).

of β-lactamase secreted by the *L. lactis* in vivo, the in vitro data was used to calculate the relative oral bioavailability. The in vivo MAT value by the *L. lactis* system was 17.8 h. This result implies that the major absorption took place within 24 h, indicating that after 24 h either there was not much more secretion of β-lactamase by the *L. lactis* or the *L. lactis* already passed through the major absorption area (the small intestine). We believe that both were possible since the *L. lactis* are transient bacteria in the GI tract, and the nutrients become less and less when the *L. lactis* move down along the GI tract so that they cannot produce much β-lactamase. From the in vitro β-lactamase secretion profile, it can be seen that the accumulated β-lactamase did not increase very much between 24 and 72 h. Therefore, it seems reasonable to use the 24-h accumulated amount (1250 mU) as an estimate for the in vivo dose. This accumulated amount of β-lactamase was secreted by 3×10^7 *L. lactis* in 3 ml of M17 broth plus the replacement of total 0.6 ml× 7 of the fresh M17 within 24 h. It seems that the result from this in vitro condition would not underestimate much the in vivo dose, although a more accurate measurement should be pursued in the future. According to this value, the in vivo relative bioavailability was 16.7%. Thus the transformed *L. lactis* significantly ($p < 0.01$) increased the oral bioavailability by 178 and 255% as compared to that by the free solution with or without the untransformed *L. lactis*, respectively. Our previous in vitro studies demonstrated that there was a 100% increase in the β-lactamase transport across the Caco-2 monolayer when delivered by the transformed *L. lactis* as compared to the free solution form (Shao and Kaushal, 2004). The increase in oral bioavailability by the *L. lactis* is most probably due to the concentrate of β-lactamase on the absorption epithelium and the prolonged contact between the two, which were resulted from the adhesion of *L. lactis* with the GI epithelium and continuous secretion of β-lactamase by the *L. lactis*. Many studies have shown that normal flora like *L. lactis* can adhere to the mucosa through various mechanisms such as surface fibrils, protruding nature and surface adhesive molecules (Tannock, 1992). After oral administration, the *L. lactis* would grow in the GI tract and adhere to the intestinal epithelium. Thus, these bacteria would secrete β-lactamase directly onto the GI epithelium, forming a concentrate of β-lactamase on the absorption surface to increase the absorption and reduce the exposure of β-lactamase to the hostile environment in the GI tract so that degradation of β-lactamase in the GI tract would be reduced. Adhesion of the *L. lactis* also caused the bacteria to move along the GI tract at much slower pace than other content (such as food) in the GI tract. This provided a sustained delivery mechanism for β-lactamase and also prolonged the contact time of β-lactamase with the absorption surface. The sustained delivery mechanism can be seen from both the prolonged MRT and MAT. Consequently, the absorption of β-lactamase was significantly enhanced.

4. Conclusion

Compared to free solution delivery, *L. lactis* significantly increased the oral bioavailability as well as the residence time of β-lactamase in rats. Therefore in conclusion, probiotics such as *L. lactis* when transformed by special plasmids can be a living source for efficient and sustained oral delivery of protein drugs.

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