IJAS Iranian Journal of Applied Animal Science

Genetic Transformation of Amylase Gene to Ruminal *Bacteroides* Species Using Conjugation Consequence for Improvement of Rumen Enzyme

Research Article

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Received on: 27 Dec 2015
Revised on: 7 Feb 2016
Accepted on: 15 Feb 2016
Online Published on: Sep 2016

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Online version is available on: www.ijas.ir

ABSTRACT

Rumen bacterial strains can potentially be manipulated to perform functions different from wild type species. The most numerous species of bacteria in the rumen and gut are species of the family Bacteroidetes, which can have the potential for genetic modification for enzyme production. One of the genetic manipulation of rumen bacteria can perform for production of starch digestive enzyme for the enhancement of nutrient flow to the rumen. In this study, *Bacteroides* species were isolated from rumen of cows. The 16S rRNA gene analysis was used to confirm the classification of the *Bacteroides* species. The amylase gene from *Bacillus* spp. was cloned into expression vector pET28A and then subcloned in *Escherichia coli-Bacteroides* pGFK114.1 shuttle vector. Conjugation between *Escherichia coli* and a *Bacteroides* strain was accomplished with two *Escherichia coli* donors containing pGFK114.1 and pRK231 and one *Bacteroides* strain as a recipient. Transfer of amylase gene by vector was confirmed using a marker cefoxcitin antibiotic resistant gene. The amylase activity assay showed that successful expression of enzyme occurred in ruminal *Bacteroides* species.

KEY WORDS 16S rRNA gene, *Bacteroides* spp., conjugation, manipulation, shuttle vector.

INTRODUCTION

Enzyme supplementation in diets for ruminants has been shown to improve growth performance, but the cost of enzyme development and production has restricted the availability of effective products and prevented the widespread use of enzymes as livestock feed additives (Selinger *et al.* 1996). Ruminants are known to harbour a vast and diverse microbial community that functions in utilizing the fibrous and starchy feedstuffs. The microbial fermentation of fibrous and starchy feed is carried out by different groups of microbiota, which function in synergistic mechanism. The exploration of the shift in carbohydrate utilizing microbial community with the change in diet, will reveal the efficient role of that group of microbial community, in particular for carbohydrate utilization. The understanding of carbohydrate utilization in ruminants is now expanding with the advent of molecular technologies (Parmer *et al.* 2015). Over the last decades however, developments in recombinant DNA technology have increased the efficiency of existing microbial production systems, and facilitated the exploitation of alternative sources of digestive enzymes (Selinger *et al.* 1996).

Competition in the animal industry has increased and for this reason some producers are interested in applying recombinant DNA technology to bacteria with rumen origin (Smith and Hespell, 1983). Early strategies on the genetic manipulation of rumen bacteria for production of digestive enzyme, focused on the enhancement of nutrient flow to the rumen by increasing fiber digestion from plant tissues,

without paying much attention to starch digesting activities (Smith and Hespell, 1983; Hespell, 1987). Starch is a major component in diets fed to intensively raised cattle especially in finishing beef cattle, where it provides the majority of the animal's energy intake (Tricarico *et al.* 2007).

Numerous animal digestion studies have shown that rumen starch digestion is somewhat incomplete, particularly in diets of high grain composition fed to beef animals having high feed intakes Hespell (1987). Starch is an important component of the ruminal diet, and its digestion is essential for maximum productivity.

The potential benefits of amylase include better feed efficiency, lower feed cost, higher weight gain and living health, lower fecal volume and nitrogen excretion. It has been reported that the amylase activity increased milk production in lactating dairy cows (Krause *et al*. 1989; Boyles *et al*. 1992; Tricarico *et al*. 2005), as well as increasing protein, reduced fat and the molar proportion of propionate in dairy cattle (Tricarico *et al.* 2007). Improvements in energy balance in transition dairy cows fed α-amylase resulted in increased weight gain and improved carcass characteristics (Defrain *et al.* 2005).

Also α-amylase has been shown to modify ruminal fermentation and improve milk yield and its component in lactating Holstein cattle (Tricarico *et al*. 2005). The results from Heinrichs *et al.* (2007) suggest that amylase may enhance rumen tissue growth when fed in calf starter. Klingerman *et al*. (2009) reported that adding exogenous amylase to a normal starch diet increased milk yield in dairy cows. Bacteria are the most important microbes involved in ruminant digestion, and there are different types in the rumen. A large proportion of rumen bacteria belong to *Bacteroides* (Prescott *et al.* 2005).

Bacteroides species are abundant members of the rumen and gut community. *Bcteroides* are obligately anaerobic, Gram-negative, non sporulating, rod shaped bacteria (Avgustin *et al*. 1994). Transformation in *Bacteroides* is not so easily done, since gene transfer methods like transformation and transduction may result in problem in the rumen, whereas conjugation has more application among rumen bacteria (Russell and Wilson, 1988).

There is a report of transfer gene by conjugation between *E. coli* and *Bacteroides* species. The clone xylanase gene was transferred from *E. coli* to two *Bacteroides* species, *B. fragilis* and *B. uniformis* by conjugation involving a shuttle vector plasmid (Whitehead *et al.* 1990). Some work has indicated that antibiotic resistance genes can be moved from *E. coli* to *Bacteroides* by conjugation (Russell and Wilson, 1988). Another report of gene transfer into ruminal bacteria refers to plasmid RB4 being moved from *E. coli* to *Bacteroides fibriosolvent* in a coculture (Teather *et al.* 1984).

In present study, we describe the cloning of an α-amylase gene from a *Bacillus* strain; transfer via a shuttle vector into a ruminal anaerobe strain of *Bacteroides* and finally amylase activity showed after conjugal transfer.

MATERIALS AND METHODS

Bacterial strains, plasmids and culture condition

Bacterial strains and plasmids used in this study are listed in Table 1. Plasmid pGFK114.1 and pRK231 were kindly supplied by Dr. Nadja Shoemaker, University of Illinois USA and α-amylase gene cloned in pET28a was a kind gift from Dr. Khosro Khajeh, Tarbiat Modares University, Iran. The *E. coli* strains DH5α and TG1 (purchased from Pasteur Institute, Iran) were grown on LB medium with antibiotics for selection of transformants. *Bacteroides* species were screened from cow's digestive system fluid and were grown on TYG (trypticase, 5g/l; yeast extract, 2.5g/l; glucose 1g/l; Sigma) or Brain Heart infusion, Difco (BHI) medium with gentamicin (200 µg/mL) in an anaerobic condition under 100% CO_2.

Cloning procedure

α-amylase gene was isolated from *Bacillus* spp. KR8104, cloned in pET28a.

Primer pairs (F-*NcoI*: 5'-CATGCCATGGCGCCATCAATAAAGAGCGGGACG-3' and R-*SacI*: 5'- TTCGAGCTCGATGGGGAAGAGAACCGCTTAA-3') with restriction enzyme sequences at the 5′ end of them were designed for amplification of amylase gene according to the multiple cloning site of shuttle vector pGFK114.1. PCR was performed using pfu polymerase to amplify 1850 bp of the amylase gene. For making sticky ends of PCR products and shuttle vector, double digestion was done using *NcoI* and *SacI* restriction enzymes. DNA was purified using DNA extraction kit (Roche®, Germany).

Digested amylase genes were inserted into shuttle vector pGFK114.1 using T4 DNA ligase (TakaRa®, Japan) and incubated at 16 ˚C for overnight. The resulting vectors were transferred to *E. coli* DH5α by heat shok transformation and grown on LB plates with X-gal and IPTG as well as ampicillin (100 µg/mL) for selection of transformants. Blue and white colonies observed after *E. coli* growth on plate. A single and clear blue (negative) and white (positive) colony was selected for further study. The positive clone was examined using standard techniques such as PCR amplification, restriction endonuclease mapping as well as DNA sequencing.

Bacteroides species screening

Bacteroides species were isolated from rumen and gut fluid of hybrid Holstein cows.

Samples were taken immediately after slaughter and maintained anaerobically in a 37 ˚C incubator. Anaerobic dilutions were spread directly into TYG agar plate and BHI supplemented with cysteine (0.5%) and vitamin K (1 mg/1000 mL) which contain gentamicin (200 µg/mL) then incubated in an anaerobic jar with gas pack at 37 ˚C for 2 days. After growing, single colonies were picked from the plates and subculture to TYG medium for additonal growth and microbiological tests (Gram staining, detection of *Bacteroides* species by 16S rDNA sequencing, anaerobic test) were performed, together with checking amylase activity.

Table 1 Bacterial strains and plasmids

Strains or plasmids	Phenotype
Esherchia coli	
DH5α	-
TG1	-
Bacteroides species	Gen^r, No amylase activity
pGFK114.1	Amp^r, Cef^r
pRK231	Kan^r, Tet^r, Tra^+
pET28a	Kan^r, inserted amylase gene

Gen: gentamycin; Amp: ampicillin; Cef: cefoxitin; Kan: kanamycin; Tet: tetracyclin and Tra, ability to self transfer.

Mating experiment

Conjugation was done by modified method of Shoemaker *et al.* (1991). Two *E. coli* strains DH5α and TG1 as donors were grown in LB broth plus antibiotics to select for pGFK114.1 and pRK231 and the *Bacteroides* recipient strains were grown in TYG medium with gentamicin (200 µg/mL) to an OD of 0.15-0.25 at 650 nm. Donors and recipient cultures (0.1 mL of each) were added to 10 mL fresh LB broth (no antibiotic) and cocultured at 37 ˚C for a few hours. The culture was then centrifuged in 10000 × g, 2 min at 4 ˚C (Sigma 3-16PK), the supernatant was removed and the resulting cell pellet suspended in 0.5 mL of fresh TYG (no antibiotic) medium to remove any residual antibiotic from the cells. After 2 min of centrifugation, the pelleted cells were resuspended in 100 µL of fresh TYG and then spread on TYG plates with 1% starch and cefoxitin (20 µg/mL) and incubated in an anaerobic jar with gaspak for 48 h at 37 ˚C. For negative control the recipient cells were subcultured alone on a selective plate.

16S rRNA gene sequencing

The *Bacteroides* species was determined using 16S rRNA gene sequencing. Primers were designed according to universal primers for *Bacteroides* spp. as previously described by Stevenson and Weimer (2007). Primer pairs were selected for regions conserved among all examples of a particular species or genus and for a much sequence divergence as possible from those of other closely related species. The 16S rRNA gene sequences of selected *Bacteroides* species were amplified, using primers.

T_m value near 60 ˚C to amplify a region of between 60-200 bp in total. The phylogenetic tree drew using phylo-draw software based on homology alignment after sequencing of 16S rRNA genes.

RESULTS AND DISCUSSION

Amplification of amylase gene and cloning in shuttle vector

The restriction enzymes sequence designed two 5' ends of primers and then PCR amplification of amylase gene was performed. A major band of amylase gene about 1850 bp was detected in 1% agarose gel (Figure 1).

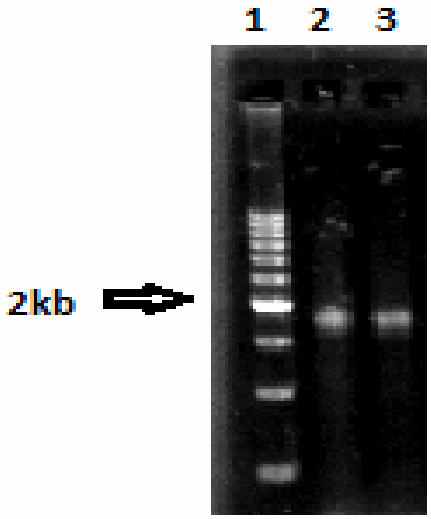

Figure 1 Alpha amylase gene amplification using DNA polymerase pfu Lane 1: DNA size marker (Sigma, 500-10000 bp, D3937) and Lane 2 and 3: PCR product of amylase gene (1850 bp)

The shuttle vector pGFK114.1 as described above was contained a *Bacteroides* promoter and ribosome binding sites, ampicillin and cefoxitin resistance genes for *E. coli* and *Bacteroides*, respectively (Figure 2).

Before cloning, the shuttle vector and amplified gene were double digested with *NcoI* and *SacI* restriction enzymes. The digested gene product was inserted in pGFK114.1 and the final cloned product with 9.68 kb was detected in agarose gel (Figure 3). The cloned product transferred to *E. coli* DH5α because some bacteria including intestinal *Bacteroides* are not so easily transformed (Russell and Wilson, 1988) and successful transformation requires shuttle vectors capable of replicating in both a rumen bacteria and *E. coli*.

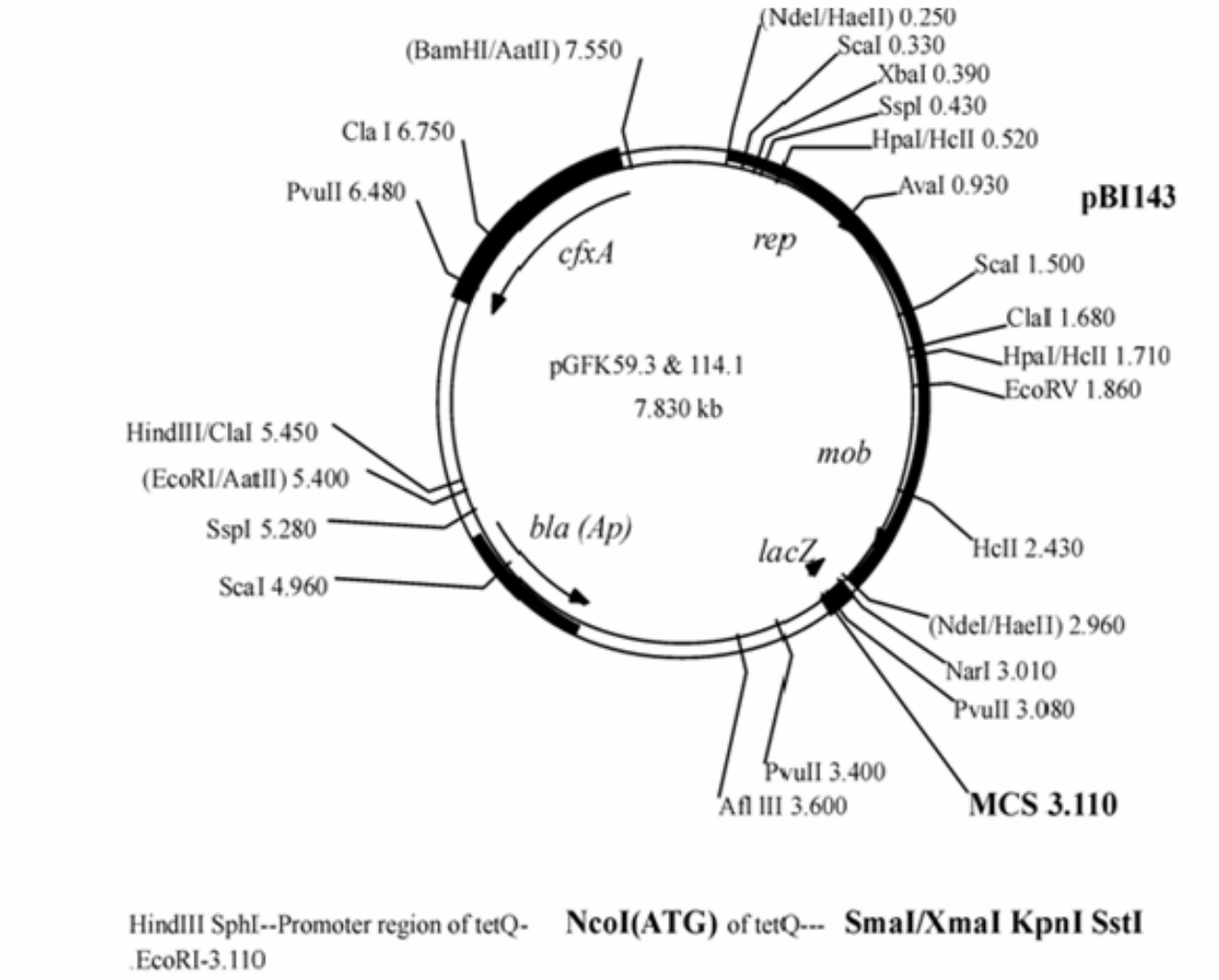

Figure 2 pGFK114.1 picture, Plasmid name: pGFK59.3 Pq inducible; pGFK114.1 Pq constitutive. Constructed by Lhing Yew Li (unpublished)
Comments: BstE11-Pst1 fragments of pLYL7 (large) and pFD160R (small) together to generate a cefoxitin resistance shuttle vector
Pq promoter sequences (280 bp) cloned into Sph1-Sst1 of pLYL05 of choice into Nco1-ATG of tet Q to the Sma1 or Kpn1 or Sst1 sites

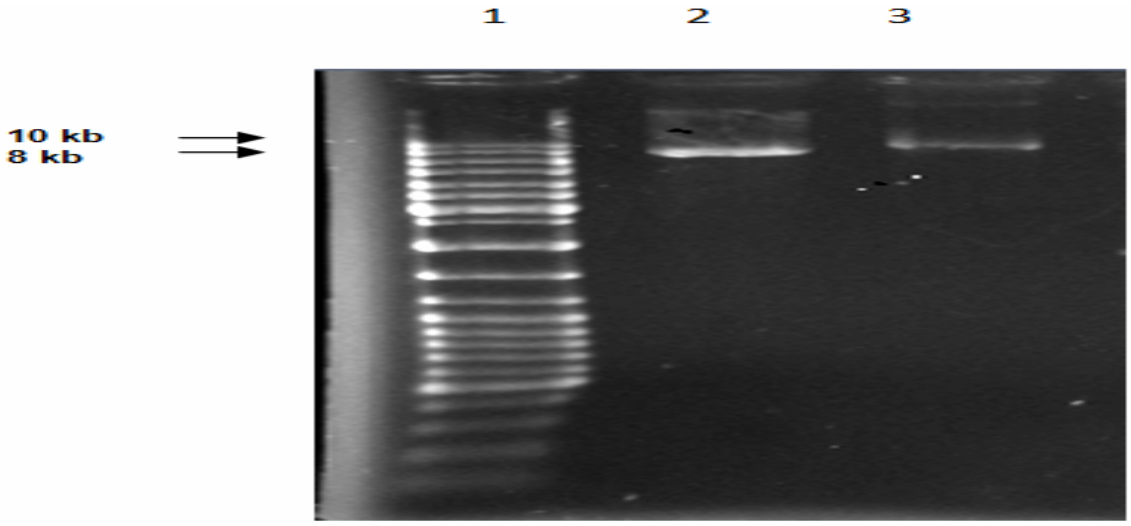

Figure 3 Cloning of amylase gene
Lane 1: DNA size marker (Fermentas, 100-10000 bp, SM0333); Lane 2: pGFK114.1 vector without insert gene (7.8 kb) and Lane 3: after cloning (9.68 kb)

The subsequent subclone was confirmed using vector digestion by restriction enzymes and PCR amplification (Figure 4) and sequence analysis of cloned gene (data was not shown). The results of these tests confirmed the presence of the gene into vector.

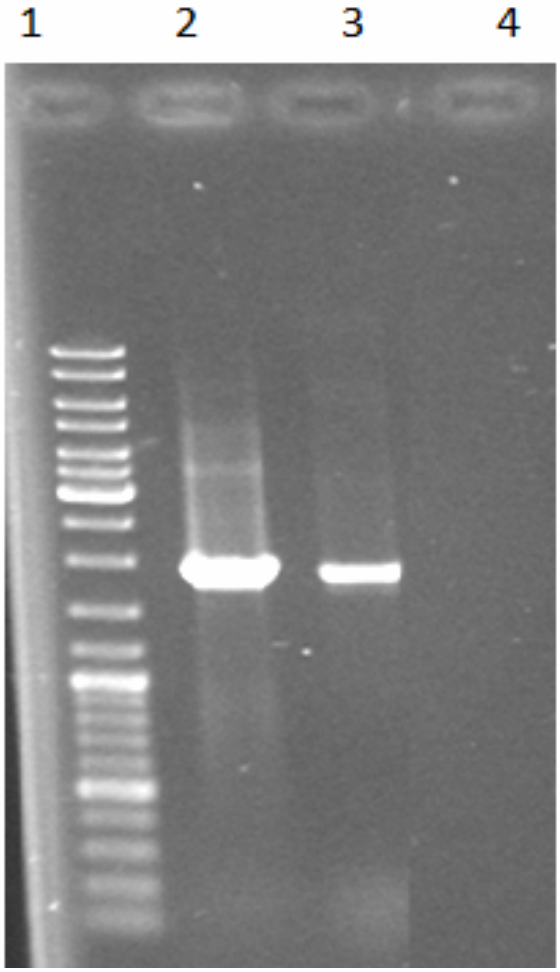

Figure 4 PCR products of cloning vector extracted from white and blue colonies

Lane 1: DNA size marker (Fermentas, 100-10000 bp, SM0333); Lane 2: ampilified amylase gene from pET28a (positive control); Lane 3: PCR product of white colony and Lane 4: PCR product of blue colony (negative control)

Bacteroides isolation and characterization

Bacteroides were isolated from rumen and gut and grown on selective media. After that any isolate was tested for Gram negative, an obligate anaerobe and rod-shaped (data was not shown). In our case, 99% of the isolates were *Bacteroides* which were then tested for amylase activity. The 16S rRNA gene sequence was used to identify the species, and the *Bacteroides* strain confirmed. The result from this analysis showed that our isolate was very close (96% identity) to *Bacteroides gallinarum* and other described strains of *Bacteroides* (Figure 5).

The match 16S rRNA gene sequence in the RDP database reflected high degree of relatedness between the target species and cultured and uncultured *Bacteroides* strains. The 16S rRNA gene sequence obtained from our *Bacteroides* spp. EH89 isolate has been deposited in GeneBank under accession no. JN627446.

Conjugation

For conjugation, we used tri-parental mating (2 donors and 1 recipient). Conjugation was done between *E. coli* a donor contains pGFK114.1 and helper plasmid pRK231 for mobilization and 10 *Bacteroides* isolates. Selection of successful transfer performed using TYG plates supplemented with 0.2% starch and cefoxitin. The amylase gene was expressed in colonic non-amylolytic *Bacteroides* species and the activity in this strain could degrade starch to glucose as bright region appeared after treatment with Lugol's solution. For a negative control unconjugated *Bacteroides* isolates were used (Figure 6).

Characterization and expression of gene encoding a variety of hydrolytic enzymes, such as cellulases, xylanases, amylases, pectinases, phytases and proteases can potentially foster the development of more efficacious enzyme expression systems for enhancing nutrient utilization by domestic animals (Selinger *et al*. 1996).

However, nutrition to an animal offers a mean to make a rapid change in milk composition i.e., concentration of milk fat, where the amount of roughage, forage: concentrate ratio, carbohydrate composition are the key factors to be taken into account (Parmer *et al.* 2015).

There have been a few genes cloned into ruminal bacteria (Wallace, 1994; Mc Sweeney *et al*. 1999) whereas there have been many reports of gene being cloned from ruminal microorganisms into other expression systems like *E. coli* (Mackie and White, 1990; Wallace, 1994).

Whereas in our work, gene transfer into ruminal microorganism was performed, and therefore rumen microorganisms characterize the expression system. *Bacteroides* are more closely related to ruminal organisms than *E. coli* and translational and post translational characteristics would be expected to be more similar (Wallace, 1994). We selected a *Bacteroides* cloning system that might be expected to be superior to the *E. coli* ones for cloning gene from ruminal bacteria.

In the present study, an amylase gene cloned in shuttle vector was transferred from *E. coli* to ruminal *Bacteroides* spp. Plasmid transfer between *E. coli* strains has also been demonstrated in the rumen (Foranoand Flint, 2000) and there are many reports about the transfer of various genes between *E. coli* and ruminal bacteria (Mackie and White, 1990; Wallace, 1994).

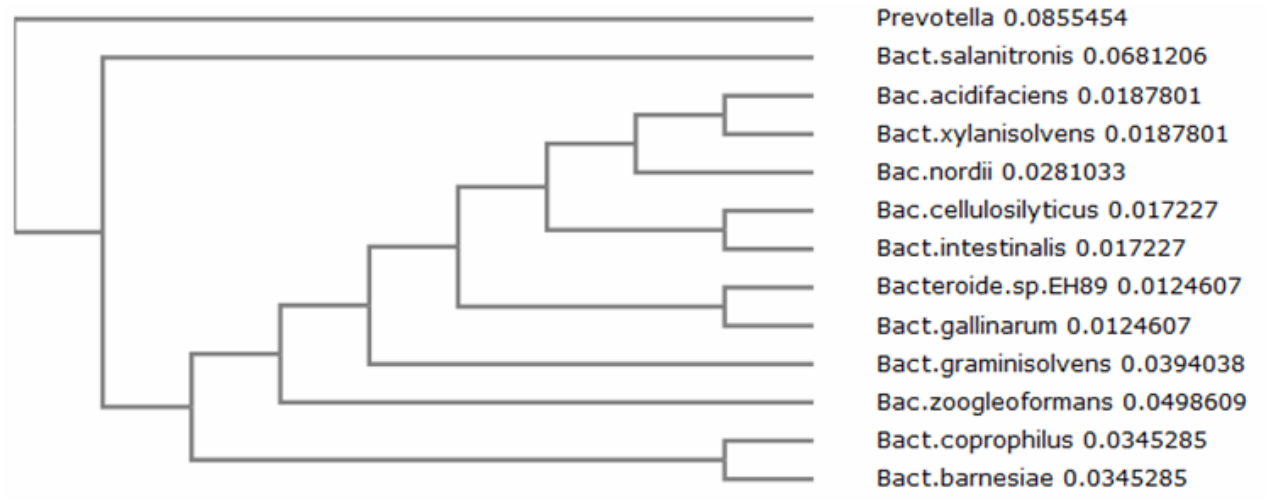

Figure 5 Phylogenetic tree of *Bacteroides* strain EH89 and related *Bacteroides* species inferred from sequence of 16S rRNA gene created by neighbor joining method
Bac: *Bacteroides*

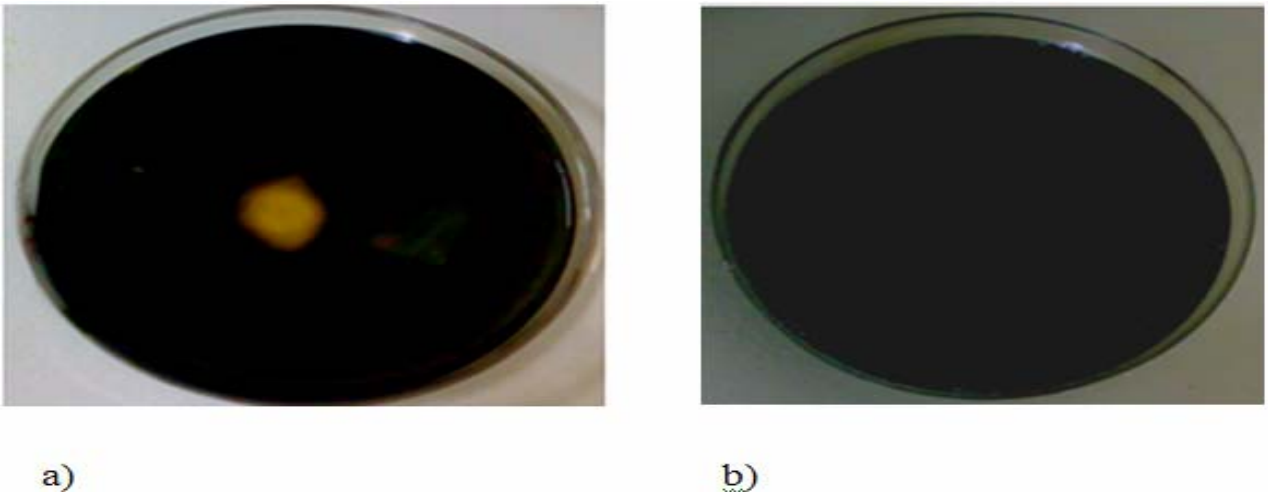

Figure 6 Amylase activity after conjugation between *E. coli* and *Bacteroides* strain EH89
a): zone of clearing in plate indicats the amylase activity after conjugation and treatment with Lugol's solution, which reacts with starch
b): negative control, *Bacteroides* spp. without conjugation

Additionally, there are reports of antibiotic resistance gene transfer and expression in ruminal bacteria by different methods of conjugation and electroporation (Hespell and Whitehead, 1991; Shoemaker *et al*. 1991; Cocconcelli *et al*. 1992; Thomson *et al*. 1992; Ware *et al*. 1992; Whitehead, 1992; Bechet *et al*. 1993). Several bacterial species have been used as recipients for gene transfer by conjugation because rumen is also considered as a potential site for natural gene transfer. Scott and Flint (1995) showed that plasmid transfer could occur between ruminal strain of *E. coli* added to whole rumen contents, while an earlier study (Smith, 1975) showed transfer of antibiotic resistance between *E. coli* strains *in vivo* in the rumen of starved cattle. Conjugation is the most widely reported mechanism of gene transfer involving ruminal bacteria.

The conjugative transfer of plasmids between species and within species has been shown in to occur *in vitro* in several species (Flint *et al*. 1988; Forano and Flint, 2000). Scott and Flint (1995) demonstrated conjugative transfer of an antibiotic resistance plasmid between two strains of *E. coli*. Flint *et al*. (1988) reported the conjugative transfer of tetracycline resistance between two strains of ruminal bacteria and Shoemaker *et al*. (1992) showed that conjugative transfer of plasmids could occur between human colonic *Bacteroides* and *Prevotella rumincola*. The promiscuous plasmid RP4 was introduced into *Butyrivbrio fibrisolvens* by conjugal transfer from *E. coli*, and the successful transfer was demonstrated by the acquisition of ampicillin resistance (Wallace, 1994). Russell and Wilson (1988) used the *B. fragilis* R751 plasmid containing a pE5-2 shuttle vector

to transfer erythromycin resistance between *E. coli* and *P. ruminicola* (Russell and Wilson, 1988). In this study, the amylase gene was transferred using shuttle vector and helper plasmid by conjugation. The shuttle vector had promoter for *Bacteroides* expression and a ribosome binding site and was also mobilized from *E. coli* to *Bacteroides sp.* using pRK231. IncP plasmid pRK231 recommend a good system for conjugal transfer of DNA from *E.coli* to other bacteria's that is an excellent mobilizer of other plasmids, including those from very distantly related bacteria (Salyers *et al*. 2000).

Plasmid pRK231, used as a helper plasmid, is a tra^+ derivative of RK2 with oriT region to facilitate conjugation in the broad host range bacteria. OriT is a short sequence of DNA that is necessary for transfer of a bacterial plasmid from a bacterial host to recipient during conjugation (Meyer and Hinds, 1982; Shoemaker *et al*. 1986; Murphy and Malamy, 1995). The advantage of conjugation to introduce foreign DNA into target organism is that if conjugation works, the plasmid can replicate in the strain of interest and the marker gene is expressed (Salyers *et al*. 2000). Therefore, we could observe the amylase activity in zymogram plate as target expressed gene. Starch is an important component of the ruminal diet and its digestion is essential for maximum productivity. However there have been a few genes cloned into ruminal bacteria and the benefit of cloning the amylase gene is associated with better feed efficiency, lower feed cost, higher weight gain and living health.

CONCLUSION

This is the first report of amylase gene transfer into ruminal bacteria isolate. This amylase gene had stability at low pH and fairly high temperature and has characteristics compatible with the ruminal environment. We found that plasmid pGFK114.1 can be transferred using conjugation from *E. coli* to *Bacteroides*. These results offer direct evidence that exchange of genetic material between the distantly related *E. coli* and *Bacteroides* spp. is possible and may actually occur in normal habitats such as the ruminant digestive system. Also, the information gained by this study can be helpful in developing carbohydrate utilization for the better nutrition of livestock.

ACKNOWLEDGEMENT

We are grateful to Dr. Nadja Shoemaker for providing the *Bacteroides E. coli* shuttle vector pGFK114.1 and IncP-plasmid pRK231 and also Dr. Khosro Khajeh for pET28a vector. This original research was performed in Sari Agricultural Science and Natural Resources University, Department of Animal Science. We thankful for providing facilities and assistance.

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