VARIATION IN THE PRESENCE OF PLASMIDS ASSOCIATED WITH PROTEINASE AND BACTERIOCIN PRODUCTION OF *LACTOCOCCUS LACTIS* ISOLATED FROM NATURALLY FERMENTED MILK

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Abstract

***Lactococcus lactis* is a major member of the lactic acid bacteria and it is known to produce bacteriocins and proteinases. A total of 32 *L. lactis* strains were isolated from naturally fermented raw cattle milk and skimmed milk. Only 4 isolates had correlative proteinase and bacteriocin production abilities with evident zones of inhibition and zones of proteolysis (above 5 mm diameter). *L. lactis* RCM9 had 3 small sized plasmids (1 kbp, 3.5 kp, and 4.5 kbp). *L. lactis* RCM 21 also possessed 3 plasmids (6 kbp, 9 kbp, and > 10 kbp), while *L. lactis* SKM 14 possessed 1 plasmid > 10 kbp and *L. lactis* RCM 15 possessed no plasmid. A combination of plasmid curing treatments of novobiocin (10 µg/ml), ethidium bromide (40 µg/ml), and incubation at 40°C resulted in an entire loss of plasmids. Phenotypic assertions on the plasmid related bacteriocinogenicity and proteinase activity of both plasmid cured and uncured isolatesrevealed thatbacteriocin and proteinase production in *L. lactis* RCM 9 and *L. lactis* RCM 15 were not plasmid mediated but chromosomal associated. However, plasmids were responsible for similar traits in *L. lactis* SKM14 and *L. lactis* RCM 21. This showed the inconsistency in plasmid presence and activity within the strains.**

Keywords: Plasmids, bacteriocin, proteinase, *Lactococcus lactis*, fermented milk

Introduction

Lactic acid bacteria (LAB) are one of the most industrially important organisms known to man. A major importance of LAB that has given it this level of industrial value is the productionof enzymes and antimicrobial metabolites. Of all the antimicrobial substances produced by LAB, bacteriocins have been accorded the most attention. This is largely due to their species-specific inhibition and unique molecular mechanisms of action. Quantifiable evidence by earlier investigators has proven the efficacy of bacteriocins from

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LAB against an array of both Gram positive and Gram negative organisms (Suskovic *et al*., 2010). Enzyme production in LAB is also of major relevanceas enzymes like proteinases have been derived from the LAB metabolism (Hill and Gasson, 1986; Kok, 1987; Kojic *et al*., 1991). Several genera make up the LAB group, and each has unique peculiarities. One of the primary genera of LAB is the Lactococcus. This genus was formerly grouped with the streptococci. These organisms are employed as dairy starters in the manufacture of fermented milk. *Lactococcus* species also produce bacteriocins (antimicrobial peptides) like nisin, lactococcin, and diplococcin which are of great industrial importance (Geiss *et al*., 1983). Studies on the genetic mechanisms of bacteriocin production in LAB, have illuminated the role of plasmids. With respect to the industrial value of the bacteriocins produced by lactococci, *L. lactis* has been shown to be the most bacteriocinogenic of all *Lactococcus* species (Kojic *et al*., 2005).

According to Gasson (1983), plasmids can be found in strains of lactococci, and have different weights. In this same regard, some lactococci have also been found to contain no plasmids (Nissen-Meyer *et al.,* 1992). To gain a favourable insight into the lactococcal plasmid biology, 2 mechanisms have been identified to be responsible for their replication – theta replication (Kiewiet *et al*., 1993) and rolling circle replication (Leenhouts *et al*., 1991). Khan (1997) presumptively stated that the rolling circle mechanism was restricted to relatively small plasmids whose functions still remained unclear. As earlier stated, a number of lactococcal strains produce bacteriocins. Geiss *et al*. (1983) conducted an extensive survey and showed that 5% of atotal of 280 tested lactococcal strains produced bacteriocins. Among the bacteriocins identified isnisin, the most prominent of all peptide antimicrobials. Further studies on the plasmid-mediated nisin production revealed the activities of 2 chromosomally located conjugative transposons (Tn 5301 and Tn 5276) identified tobepresent in *L. lactis* and as responsible for the production of the peptide (Dodd *et al*., 1990; Horn *et al*., 1991; Rauch and de Vos, 1992). In view of the conjugative property of plasmids, the abilityfor *L. lactis* to produce other bacteriocins is possible as the plasmids for bacteriocins like diplococcin or lactococcins have proven to be transferable via conjugation (Harmon and McKay, 1987). Other studies on bacteriocin production by different strains of *L. lactis* have revealed the presence of the lactococcin A gene cluster from the plasmid pNP2 (Stoddard *et al*., 1992) and the presence of 3 different bacteriocins, namely lactococcin A, lactococcin B, and lactococcin M/N in *L. lactis* subsp. *cremoris* 9B4 (Van Belkum *et al*., 1991)

The presence of lactococci in milk can be attributed to their ability to metabolise the proteins and other compounds inherent in the environment. To ably convert the protein, a compact proteolytic system has been known to be responsible. Kojic *et al*. (2005) explained that cell-wall-bound extracellular proteinases are implicated in the crucial role of initially hydrolysing the proteins. Genetic studies by Kok (1990) and Nissen-Meyer *et al*. (1992) identified that the gene coding for extracellular proteinase production inlactococci was located on a plasmid.

This work thus seeks to investigate the abilities of plasmids in *L. lactis* to code for protein hydrolysing enzymes and also forbacteriocin production. With focus on the possible genetic relationship between the phenotypic traits of bacteriocin and proteinase production as obtained in the plasmids, this study molecularly illuminatestargets for scale-up optimisation and maximisation of the industrial potentials of LAB. The inductive effects of proteins and protein based environments on protein hydrolysis were considered as milk was used as the primary isolation environment of *L. lactis* used in the study.

Materials and Methods

Isolation and Characterisation of *L. lactis*

Freshly drawn rawcattle milk and commercially

available skimmed milk were obtained in containers and allowed to undergo natural fermentation for 72 h. Serial dilutions were made and 1ml of 10-5-10-10 as aliquots were aseptically plated out on MRS agar and M17 agar media via the pour and spread plate technique. Aerobic and anaerobic methods of incubation were observed and suspected LAB colonies were streaked onto the surface of the agar media to obtain pure cultures. To characterise the isolates, macroscopic observations and physiological characterisation included parameters such as colony shape, size, elevation, type of growth, pigmentation, spore staining, catalase test, nitrate reduction, growth in different sodium chloride concentrations, growth at different temperatures, carbohydrate fermentation tests, motility, and homolactic and heterolactic fermentation properties. *L. lactis* isolates were identified after the series of biochemical tests and screening and subsequently subjected to antagonistic studies.

Preliminary Antagonistic Studies

To ascertain the preliminary antagonistic state of the *L. lactis* isolates, the isolates were prepared in broth and aliquots were dispensed into a sterile bottle. Bacteriocin sensitive *Lactobacillus casei* 049 was obtained from the Federal Institute of Industrial Research, Lagos, Nigeriaand used as the test organism. The agar well diffusion method of Tagg and McGiven (1971) with modifications by Ajunwa (2011) was carried out. The *L. lactis* in the broth was transferred in 50 µl quantities into the wells. After incubation for 48 h, the plates were observed for zones of inhibition. Plates with at least 5 mm diameters were used and their labelled stocks were selected for assay for bacteriocin production.

Assay for Bacteriocin Production

To detect bacteriocin production, broth culture of antagonistic *L. lactis* was freshly prepared and incubated overnight at 30°C. With modifications to the procedure of Corsetti *et al*. (2004), liquid broth was centrifuged at 10000 rpm for 5 min. M17 soft agar (0.7% w/v) was used to propagate the bacteriocin sensitive *Lb. casei* 049. Wells were made in the lawn of soft agar, and 50 µl aliquots of filtered supernatant of the overnight culture were poured into the wells. The appearance of a clear zone representing inhibition of growth of the sensitive *Lb. casei* around a well implied a positive signal forbacteriocin production. Filtered supernatants of the overnight culture of bacteriocin non-producer cells of *L. lactis* were used as a negative control.

Assay for Proteinase Activity

To test proteinase activity, modified methods of Kojic *et al.* (2005) were adopted; all isolates of *L. lactis* were induced by growing them on milk-citrate agar plates containing 4.4% reconstituted skimmed milk, 0.8% Na-citrate, 0.1% yeast extract, 0.5% glucose, and 1.5% agar (w/v). The cells were grown in broth of the same medium, and the detection of caseinolytic/proteinolytic activity was performed using a casein-based medium for conduction of the agar well diffusion assay. Any form of clearing around the colonies implied apositive signal for proteinase production. They were measured and representative cultures were properly labelled.

Plasmid Isolation and Plasmid Curing

Plasmid isolation was carried out according to the method of Anderson and McKay (1983) with adaptations to the method of Jamuna *et al*. (2010). The presence of plasmids was checked via agarose gel electrophoresis with 0.7% agarose in the presence of a Tris-borate buffer at 100 volts for 4 h. Ethidium bromide (0.5 µg/ml) was used for staining the gel which was subsequently viewed under UV light on a transluminator and photographed. The plasmid size was determined according to the method of Jamuna *et al*. (2010) using the logarithmic conversion of the molecular weight of standard DNA molecular size markers run concurrently with their respective mobility.

Plasmid curing was conducted with modifications according to the method of Kojic *et al*. (2005). It was achieved by growing the cellsin pre-warmed M17 broth (40°C) containing ethidium bromide (40 µg/ml) andnovobiocin (10 µg/ml). The cells were collected by centrifugation every 2 h after incubation at 40°C. The collected cells were resuspended in the same volume of M17 broth containing novobiocin and ethidium bromide, and the process was repeated 5 times. Subsequently, 0.1 ml aliquots of cultured broth were plated on M17 agar platesand incubated at 30°C for 48 h. After incubation, plates were overlaid with M17 soft agar containing indicator *Lb. casei* 049 and incubated overnight at 30°C. Colonies were checked for inhibition against the indicator organism. Earlier identified bacteriocin producing cells without zones of inhibition after curing were termed as 'cured'. Bacteriocin production was carried out with the cured and uncured cells

Bacteriocin Production

For inducement of bacteriocin production, a chemically defined and modified MRS broth (Ajunwa, 2011) containing a reduced concentration of peptone (0.5% w/v) and glucose (0.25% w/v) was used to propagate the LAB strains at 30°C for 72 h. An anaerobic environment was also employed during incubation. Cells were removed from the stationary phase of the growth broth medium by centrifugation at 10000 rpm for 10 min at 4°C. The supernatant fluid was adjusted to pH 6.5 and then treated with 5 mg/ml catalase. This was to check the interfering effects of non-bacteriocin antimicrobial metabolites. The supernatant was then filter sterilized through a 0.4 µm pore size membrane filter and the product was designated as 'crude bacteriocin'. Filtered supernatant of the cultures of bacteriocin-non-producer cells was used as a negative control. Samples of the extracted bacteriocin fluid were subjected to the action of Proteinase K (0.5 mg/ml) to ascertain the protein nature of the fluids.

Propagation of Plasmid Cured Isolates

The plasmid cured isolates of *L. lactis* were repropagated on M17 agar and MRS agar and subjected to assay for bacteriocin production. They were also subjected to a proteinolytic enzyme assay. The resulting zones of inhibition were subsequently measured.

Ressults

Macroscopically, the *L. lactis* colonies were cream coloured on MRS medium with

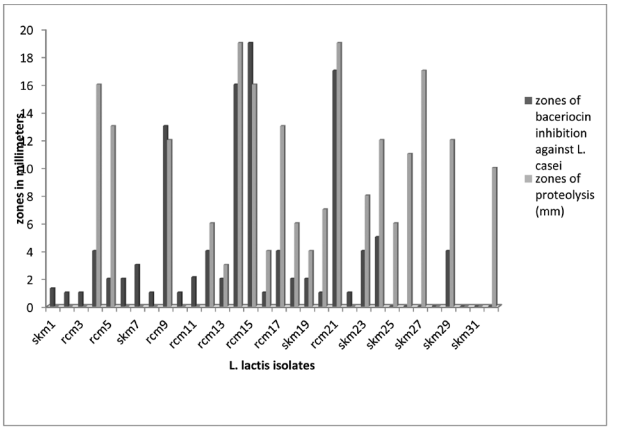

Figure 1. Zones of inhibition/zones of proteolysis of *L. lactis* isolates

smooth surfaces. The edges were entire, and the isolates were translucent. The colonies were small and round. Microscopic analysis showed the isolates to be cocci with positive Gram reaction. They were negative to nitrate reduction tests, spore tests, catalase tests, and grew in 5% NaCl. They also grew at 10°C but no growth was observed at 45°C. Biochemically, they utilised glucose, maltose, sucrose, mannitol, galactose, lactose, and xylose, but were unable to utilise arabinose. They were homofermentative in nature producing lactic acid as the primary metabolic product.

Thirty two strains of *L. lactis* were isolated from bothraw cattle milk and skimmed milk that were made to undergo natural fermentations. Isolates fromfermented cattle milk were designated with raw cattle milk (RCM) while skimmed milk (SKM) was used for isolates from fermented skimmed milk. All the isolates were subjected to primary antagonistic studies against *Lb. casei*, and proteinase assay. Twenty of the isolates were positive for proteinase production. Out of the total number, only 4 isolates showed zones of inhibition (above 5 mm) against the test organism which was indicative for bacteriocin production. The 4 isolates were also positive for proteinase production, indicating a phenotypic correlation in proteinase and bacteriocin expressions. Figure 1 shows the zones of inhibition (mm) of the *L. lactis* isolated from the 2 milk sources against non-bacteriocinogenic *Lb. casei* as test organisms, and zones of clearing (mm) of proteolysis. Plates showing phenotypic expressions of bacteriocin and proteinase activity were illustrated by Figures 2 and 3.

Plasmid profiling carried out on the 4 bacteriocinogenic and proteinase producing *L. lactis* isolates revealed that *L. lactis* RCM 9 possessed 3 plasmids of approximately 1 kbp, 3.5 kbp, and 4.5 kp. *L. lactis* SKM 14 possessed a plasmid greater than 10 kbp. Three plasmids were present in *L. lactis* RCM 21 with weights of 6 kbp, 9 kbp, and > 10 kbp, while *L. lactis* RCM 15 possessed no plasmid. Plasmid curing conducted on all 4 isolates showed a complete elimination of all plasmids which was evident by the loss of plasmids after a plasmid profile was re-conducted. Figure 4 shows the plasmid profile of all 4 *L. lactis* isolates.

Plasmid cured isolates were subjected to phenotypic determinations of bacteriocin activity and proteolysis. It was observed that plasmid cured *L. lactis* RCM 9 and *L. lactis* RCM 15 still showed bacteriocin and proteolytic activity, proving that the bacteriocin and proteolytic activities were not plasmid mediated. However, *L. lactis* SKM 14 and *L. lactis* RCM 21 showed negative bacteriocin

Figure 2. Plate showing zones of inhibition of bacteriocin fluids from *L. lactis* isolates against non bacteriocinogenic *L. casei*

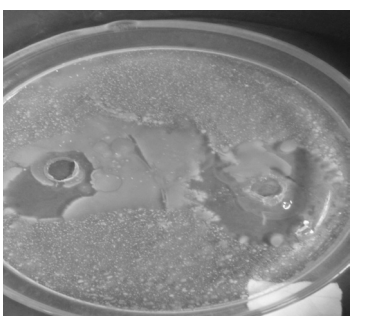

Figure 3. Plate showing the zones of proteolytic clearing by proteinase producing *L. lactis* isolates

and proteolytic activities after plasmid curing proving the fact that the traits were plasmid associated and probably plasmid mediated.

Discussion

The isolation environments (raw cattle milk and skimmed milk) generally aided the luxuriant growth of *L. lactis* with no specific differences in the expressional abilities of bacteriocin and proteinase activities of the isolates derived from the 2 environments. The results from this work showed that there was a correlation in the expression of proteinase activity with bacteriocin mediated inhibition in some *L. lactis* strains. The effects of plasmids in relation to phenotypic characters has thrown open a major avenue of inquiry in molecular biology, especially with respect to bacteria of industrial importance. This has proved to be in direct concord with reports by Sieuwerts *et al*. (2008), and Suskovic *et al*. (2010). However, the interplay in plasmid mediation of these characteristics canbe based on factors like the size of plasmids and the number of plasmids present within a cell, as evidenced in analysis by Turner (2004).

Plasmids occurring in the bacteriocin and proteinase producing cells of the *L. lactis* isolates ranged from 1 kbp to > 10 kbp. *L. lactis* RCM 9 which possessed the small sized plasmids (1 kbp, 3.5 kbp, and 4.5 kbp) proved to possess bacteriocin and proteinase production traits independent of the plasmid possessed. This case proves that the plasmids did not concern both bacteriocin and proteinase production, and that the bacteriocin and proteinase production genes were chromosomally located. With regards to the small size of the plasmids observed in the isolate, Khan (1997) explained thatboth the existence of relatively small lactococcal plasmids and their functions have been relatively unclear. *L. lactis* RCM 15 possessed no plasmids and thus its bacteriocinogenicity and proteinase production traits were not affected by plasmid curing. This confirms reports about the unsteady genetic configuration of traits like bacteriocin production in *Lactococcus* species (Nettles and Barefoot, 1993).

L. lactis SKM 14 and *L. lactis* RCM 21 possessed bacteriocin and proteinase production

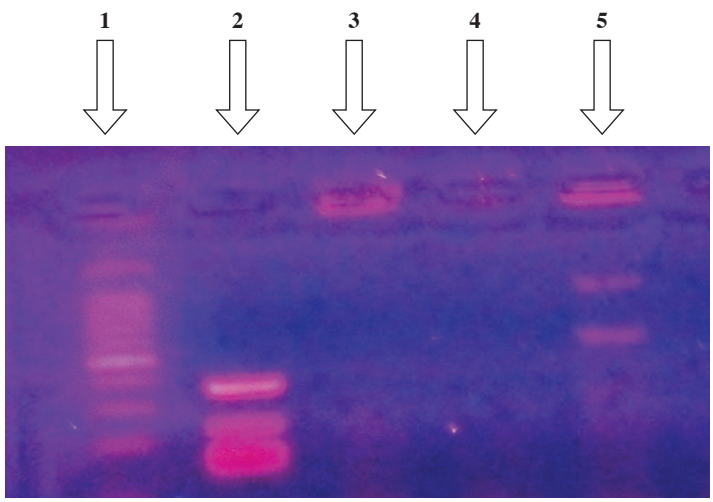

Figure 4. Plasmid profile of bacteriocin and proteinase producing *L. lactis* isolates

Lane 1: 10 kbp DNA ladder
Lane 2: *L. lactis* RCM9 (plasmids: 1 kbp, 3.5 kbp, 4.5 kbp)
Lane 3: *L. lactis* SKM14 (plasmids: > 10 kbp)
Lane 4: *L. lactis* RCM15 (plasmids: nil)
Lane 5: *L. lactis* RCM 21 (plasmids: 6 kbp, 9 kbp, > 10 kbp)

traits which were plasmid mediated. They harboured plasmids of varying molecular weights, but common to both were plasmids > 10 kbp. This is in line with the findings by Kojic *et al*. (2005) which showed *L. lactis* isolates possessing very large plasmids (> 100 kilo bases) coding for proteinase and lactococcin production. The sizes of the plasmids recorded in this work are relatively small compared to those obtained by Kojic *et al*. (2005); however, further work on the specific characterisation and classical determination of the proteinolytic enzymes and bacteriocins produced (based on molecular weight determination and purification methods) will aid in conclusive exposition of the phenotypic traits of plasmid bearing *L. lactis* strains with respect to the plasmid size.

In general, plasmids are highly unstable and the clarity in their reported functions has been plagued by a plethora of laboratory and natural factors. Nevertheless, conscious attempts to preserve the plasmid-mediated beneficial traits of industrially important bacteria are consistently in motion, thus requiring extended research.

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