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Full Length Research Paper

ISOLATION AND CHARACTERIZATION OF THE BACTERIA *PHOTORHABDUS SPP*. (ENTEROBACTERIACAE) ISOLATED FROM ITS SYMBIOTIC NEMATODE PARTNER

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ABSTRACT: *Photorhabdus* bacteria are symbiotically associated with entomopathogenic nematodes of the genus *Heterorhabditis*. These bacteria produce a host of toxins that are lethal to insects and consequently they have emerged and are trending as excellent biological control agents against insect pests of agricultural crops. The nematode partner acts as a vector to carry the bacteria from one susceptible host to another. The nematode and bacteria work together to overcome the immune response of their insect host, thus allowing the bacteria to proliferate produce toxins that kill the host insect. The bacteria was isolated from the haemolymph of nematode infected *Galleria mellonella* larvae. The nematodes were isolated from soil samples collected from the study area using an established *Galleria mellonella* bait method described by Kaya and Stock, (1997). The bacteria was identified using phenotypic characters and the most important biochemical characteristics of the genus *Photorhabdus*. The isolation of this bacteria in the study area is an indication of its potential usage together with its nematode partner as biological control agents of pests of crops in Nigeria.

Key words: *Photorhabdus* bacteria, entomopathogenic nematode, biological insecticides, *Galleria mellonella,* Nigeria.

INTRODUCTION

Xenorhabdus and *Photorhabdus* species of bacteria have emerged as excellent biological pesticides as well as an excellent alternative to synthetic pesticides for the successful control of insect pests of agricultural crops in some countries of the world (Burnell and Stock, 2000; Gaugler, 2002). They are non spore producing, Gram-negative, facultative anaerobic motile rods (Thomas and Poinar, 1979; Owuama, 2001). They have a mutual relationship with insect parasitic nematodes that belong to the families Steinernematidae and Heterorhabditidae respectively (Boemare, 2002; Adams *et al*., 2006; Ferreira and Malan, 2014). These bacteria according to the report of Forst *et al*., (1997), are oxidase positive. They also belong to the family Enterobacteriacae (Akhurst and Boemare, 1990). Their growth *in vitro* is very poor at optimum temperature of 25-28°C after 24h and they have never been isolated alone without their nematode hosts in the soil (Akhurst *et al*., 1992; Smiegielski *et al*., 1994). *Xenorhabdus* species are catalase negative and both *Photorhabdus* and *Xenorhabdus* species are usually unable to reduce nitrate, an unusual characteristics of bacteria in the family Enterobacteriaceae (Farmer *et al*., 1989). The relationship between the bacteria and the nematode is symbiotic. The nematode is dependent upon the bacteria for killing the host insect, creating a suitable environment for the development of the nematode, breaking down the hosts tissues into utilizable nutrients and serving as a food source. The nematode protects the bacteria from the external environment since the bacterium is unable to survive living freely (Poinar, 1979). The nematode acts as a vector, penetrates the host and releases its symbiotic bacteria into the haemocoel of the host insect (Hinchliffe e*t al*., 2010). The bacteria produces a spectrum of toxins and antibiotics (Maxwell *et al*., 1994; Hinchliffe *et al*.,2010) which inhibit potential competitors. The bacteria proliferate and provide nutrition for the developing nematodes (Poinar, 1990; Webster *et al*., 2002). The multiplication of the *Xenorhabdus* or *Photorhabdus* bacteria leads to septiceamia and toxemia and eventual death of the insect host.

MATERIALS AND METHODS

Nematodes: Nematodes were isolated from soil samples in Kashere using an established *Galleria mellonelle* bait method and the white trap.

Isolation of bacteria from infective juveniles

Isolation of infective juveniles was done according to a modification of the method of Kaya and Stock (1997).

Infective juveniles (IJs) were collected from white traps and allowed to settle under gravity in a 50ml beaker. The infective juveniles were surface sterilized with 0.1% sodium hypochlorite for 3 hours to get rid of any possible bacterial and fungal contaminants on the surface of the nematodes. The sterilized nematodes where rinsed in distilled water.

The nematodes were placed in sterile test tubes and centrifuged at 3000rpm for five minutes to burst the nematodes and release the bacteria in them into the distilled water contained in the test tube. Using a sterile wireloop some of the bacterial suspension contained in the test tube was inoculated on to nutrient agar, nutrient agar bromothymol blue and MacConkey agar plates. The plates were incubated at 32ºC for 2-3 days. Single colonies were picked with a sterile wireloop and sub-cultured twice to obtain a pure culture. The plates were stored at refrigeration

temperature until required for analysis. The bacteria isolates were sub cultured on nutrient agar slants for storage.

Isolation of bacteria from the haemolymph of *Galleria mellonella* infected with nematodes

A method decribed by Kaya and Stock, (1997). Infected *Galleria mellonella* larvae are recognized by a colour change in dead larvae from the usual cream colour of healthy larvae to dark red for *Hetrorhabditis* spp. and brown or yellow for *Steinernema* spp. usually 48hrs after death. Dead and infected larvae were then surface sterilized in 70% alcohol for 10 minutes. The larvae were allowed to dry and a fore limb was removed with a sterile scissors. Using a sterile wireloop, a drop of oozing haemolymph from each larva was streaked on nutrient agar (Oxoid) plates and the plates were incubated at 32ºC for 48hrs.

Single colonies of the bacteria was selected and streaked onto new plates of nutrient agar, and sub-culturing was done until colonies of uniform size and morphology were obtained. The isolates were stored on nutrient agar slants for further analysis and storage.

Phenotypic and biochemical characterization of bacterial symbionts

In order to identify the bacteria associated with the isolated nematode, the most important phenotypic characteristics described by Boemare and Akhurst, (1988) and modified by Ferreira *et al*., (2014) were adopted. Pure culture of the bacteria was obtained by picking distinct colonies of the bacterial isolate which were streak-plated on nutrient agar and then incubated at 32ºC for 24hrs. This was used for morphological characterization. Cell morphologies were examined under light microscopy after gram staining. Colony pigmentation was examined on Nutrient agar, MacConkey (Oxoid) and Nutrient agar bromothymol blue plates. The agar plates were sealed with masking tape and incubated at 32˚C for 24hrs. The biochemical tests carried out included; citrate, motility and Indole production after 48 hrs, catalase, proteolysis on casein agar, and oxidase activities.

Citrate utilization test. This test is based on the ability of an organism to utilize citrate as its only source of carbon. Simmon citrate agar was used to prepare slopes in bijou bottles and stored at 28ºC. Using a sterile wire loop, the bottom of the medium was stabbed with the test organism followed by streaking on the surface of the slant. The bottles were incubated at 35ºC for 48hrs. A change in colour from green to blue indicated a positive test.

***Motility and Indole test*:** Tryptone water was used for the detection of motility and indole. Using a sterile wireloop the test organism was inoculated into a sterile bijou bottle containing 3ml of sterile tryptone water. This was incubated for 48 hrs at 35ºC. Motility was observed as a result of turbidity of the medium. 0.5ml of Kovac's reagent was added and the mixture was gently shaken. Development of red colour in the surface layer was an indication of indole production.

***Catalase activity*:** This test is based on the ability of an organism to produce the enzyme catalase. Using a sterile wireloop a small portion of the test organism was picked and smeared on clean grease-free glass slides. Drops of hydrogen peroxide were added to the smear and slightly rocked. Formation of gas bubbles was an indication of a positive test for catalase. This test

differentiates *Xenorhabdus* spp. from *Photorhabdus* spp. *Xenorhabdus* spp. are catalase negative while *Photorhabdus* spp. are catalase positive.

Proteolysis on Casein agar: 250ml of sterile skim milk was mixed with equal volume of double-strength nutrient agar at 50ºC. Using a sterile wireloop, a colony of the bacteria was picked and streaked onto the medium and the plates were incubated at 35ºC for 14 days. Clear zones surrounding the growth indicated a positive test.

Oxidase test: This test is done to determine the presence of oxidase enzymes. It helps separate Pseudomonas from oxidase negative Enterobacteriaceae. Oxidase paper strips were used for this test. The culture was grown on NBTA broth and a few drops were subsequently placed on oxidase strip. The strip was observed within 20-30 seconds. There was no colour change indicating absence of oxidase activity. Development of purple colour is an indication of the presence of oxidase activity. Both *Xenorhabdus* spp. and *Photorhabdus* spp. are oxidase negative.

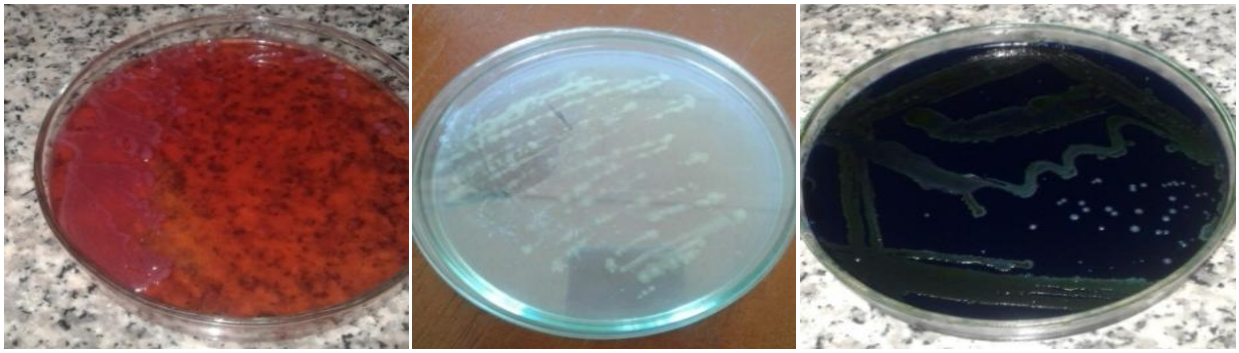
The biochemical test results such as Gram stain reaction, motility, indole production, citrate and catalase activity and proteolysis on casein agar helped to differentiate and characterize the isolates.

Results

Phenotypic characterization of bacterial endosymbionts:

Colonial characteristics of bacteria isolates were observed on nutrient agar, nutrient bromothymol blue agar (NBTA) and MacConkey agar respectively.

Colonies were 1-3 mm in diameter and they absorbed dye on MacConkey and NBTA plates. On nutrient agar they appeared milky white and swarming on MacConkey agar colonies appeared pink while on nutrient bromothymol blue NBTA plates colonies absorbed the blue colour of the dye and appeared blue-green



Pink colonies on MacConkey agar. Milky white colonies on nutrient agar. Blue-green colonies on nutrient agar bromothymol blue (NBTA).

Biochemical characterization of bacterial endosymbionts:

After gram staining, cells of the bacteria were observed under the microscope. The cells appeared as gram-negative rods. The result of biochemical tests are presentedin table below. Simmon citrate agar used for the test changed colour from green to blue indicating a positive test. The organisms produced turbidity after incubation for 48 hrs at 35ºC indicating that they are motile. The bacterial cells also produced indole when 0.5ml of Kovac's reagent was added to the mixture of Tryptone water plus bacterial cells due to the development of red colour in the surface layer. Also formation of gas bubble upon addition of hydrogen peroxide was an indication of a positive test for catalase. This test differentiates *Xenorhabdus* spp. from *Photorhabdus* spp. *Xenorhabdus* spp. are catalase negative while *Photorhabdus* spp. are catalase positive. Clear zones surrounding the growth of bacterial cells on Casein agar indicated a positive test for proteolysis. When the bacterial cells were streaked on oxidase paper strip, there was no colour change thus indicating absence of oxidase activity.

Results of Biochemical test

SN	GS	MOT	IND	CIT	CAT	PROT	OXID	NBTA	ORGANISM
Ksh1	GNR	+ve	+ve	+ve	+ve	+ve	+ve	B-G	*Photorhabdus* spp.
Ksh2	GNR	+ve	+ve	+ve	+ve	+ve	+ve	B-G	*Photorhabdus* spp.

Key: GS= Gram stain reaction, MOT= Motility, IND= indole test, CIT= citrate test, CAT= catalase, PROT= proteolysis on casein agar, OXID= oxidase test, NBTA= nutrient bromothymol-blue agar, Ksh 1= isolate number 1from Kashere, Ksh 2= isolate number 2 from Kashere, B-G= blue-green, GNR= Gram negative rods

Discussion

The bacterial endosymbionts were successfully isolated from the haemolymph of infected *G. mellonella* cadavers. The most important phenotypic and biochemical characteristics as described by Boemare and Akhurst, (1988) and that of Ferreira *et al*., (2014) were investigated and used in the identification of the bacterial isolates as *Photorhabdus* spp. *Heterorhabditis bacteriophora* is specifically associated with *Photorhabdus* species of bacteria. On nutrient agar plates *Xenorhabdus* spp. usually associated with *Steinernema* nematodes produced pale yellow colonies as against milky white colonies of *Photorhabdus* bacteria observed in this investigation. On Mackonkey agar *Xenorhabdus* produced brown colonies against pink colonies produced by *Photorhabdus* while on NBTA plates, *Xenorhabdus* produced greenish colonies with a red center as against blue-green colonies of *Photorhabdus* spp. as observed in the current study.

RESULTS

Biochemical characterization of bacterial endosymbionts:

After gram staining, cells of the bacteria were observed under the microscope. The cells appeared as gram-negative rods. The result of biochemical tests are presented in Table 2 below. Simmon citrate agar used for the test changed colour from green to blue indicating a positive test. The organisms produced turbidity after incubation for 48 hrs at 35ºC indicating that they are motile. The bacterial cells also produced indole when 0.5ml of Kovac's reagent was added to the mixture of Tryptone water plus bacterial cells due to the development of red colour

in the surface layer. Also formation of gas bubble upon addition of hydrogen peroxide was an indication of a positive test for catalase. This test differentiates *Xenorhabdus* spp. from *Photorhabdus* spp. *Xenorhabdus* spp. are catalase negative while *Photorhabdus* spp. are catalase positive. Clear zones surrounding the growth of bacterial cells on Casein agar indicated a positive test for proteolysis. When the bacterial cells were streaked on oxidase paper strip, there was no colour change thus indicating absence of oxidase activity.

Table 2: Results of Biochemical test

SN	GS	MOT	IND	CIT	CAT	PROT	OXID	NBTA	ORGANISM
Ksh1	GNR	+ve	+ve	+ve	+ve	+ve	+ve	B-G	*Photorhabdus* spp.
Ksh2	GNR	+ve	+ve	+ve	+ve	+ve	+ve	B-G	*Photorhabdus* spp.

Key: GS= Gram stain reaction, MOT= Motility, IND= indole test, CIT= citrate test, CAT= catalase, PROT= proteolysis on casein agar, OXID= oxidase test, NBTA= nutrient bromothymol-blue agar, Ksh 1= isolate number 1from Kashere, Ksh 2= isolate number 2 from Kashere, B-G= blue-green, GNR= Gram negative rods

Phenotypic and biochemical characterization of bacterial endosymbionts

The bacterial endosymbionts were successfully isolated from the haemolymph of infected *G. mellonella* cadavers. The most important phenotypic and biochemical characteristics as described by (Boemare and Akhurst, 1988) and that of (Ferreira et al., 2014) were investigated and used in the identification of the bacterial isolates as *Photorhabdus* spp. *Heterorhabditis bacteriophora* is specifically associated with *Photorhabdus* species of bacteria. On nutrient agar plates *Xenorhabdus* spp. usually associated with *Steinernema* nematodes produced pale yellow colonies as against milky white colonies of *Photorhabdus* bacteria observed in this investigation. On Mackonkey agar *Xenorhabdus* produced brown colonies against pink colonies produced by *Photorhabdus* while on NBTA plates, *Xenorhabdus* produced greenish colonies with a red center as against blue-green colonies of *Photorhabdus* spp. as observed in the current study. The rod shaped bacterial endosymbiont observed in the intestine of infective juveniles of the nematodes further confirms association of entomopathogenic nematodes with bacterial symbionts.

CONCLUSION

The key to achieving sustainable agriculture and food security in Nigeria is ensuring that crops stay healthy and protected from damage by pests and diseases. One of the ways of achieving this important goal is through the use of biological pesticides based on nematode-bacteria complex as alternative sources of pest management and control in the country. From the investigations conducted in Kashere and its environs, the presence of these important agents of biological control have been established. This is the first record of the presence of entomopathogenic nematodes-bacteria complexes from Kashere, Nigeria. The nematode partners were successfully identified as *Hetrorhabditis bacteriophora* based on important morphological characteristics of the males and infective juveniles. Colour change produced in infected cadavers were also considered and the gummy consistency of cadavers infected with *Heterorhabditis bacteriophora* were also established. The bacterial endosymbionts associated with the nematodes were also successfully isolated from the haemolypmh of infected *G. mellonella* cadavers. Important phenotypic properties like colonial characteristics of the

bacteria on Nutrient agar, MacKonkey agar and NBTA plates were observed. The symbiotic bacteria associated with the nematodes were observed in the intestine of the worms and this further confirms the nematode-bacteria complex. Biochemical characteristics of the bacteria also helped to confirm the isolates as *Photorhabdus* spp. of bacteria. The aim of the investigation was achieved. There is therefore the need to test the effectiveness of the isolate together with their nematode partners in order to establish their host for possible exploitation as biological control agents of important pests of crops in Nigeria.

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