Fishing with bait for bacterial predators in Zebra droppings - isolation and characterization of a bacteriolytic Myxococcus strain

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The isolation of bacteriolytic prokaryotes from herbivore droppings collected in South Africa (Pietermaritzburg) was undertaken by using inactivated or active microbial cells as the bait embedded in water agar. Using Zebra droppings as the source for predatory bacteria feeding on the microbial bait, a Gram negative isolate was obtained. Based on its morphological and physiological features together with its 16S rRNA gene sequence, the isolate was identified as a member of the genus Myxococcus. Light and electron microscopy revealed the typical myxobacterial life cycle involving the formation of fruiting bodies. Plate assays demonstrated that this isolate was able to lyse a range of Gram positive and Gram negative bacteria, including pathogens such as Staphylococcus aureus, Salmonella Typhimurium and Pseudomonas aeruginosa. These bacteria were utilized by the predatory isolate as sole source of carbon and energy. This shows that the simple baiting technique is a useful approach to isolate bacteriolytic microorganisms from previously untapped environmental samples which in turn could help in the isolation of new antimicrobial compounds.

Keywords Zebra dung, baiting technique, fruiting bodies, Myxococcus fulvus, bacteriolysis.

1. Introduction

Antibiotics are typically low molecular weight secondary metabolites produced by bacteria, fungi or even plants that antagonize the growth of other microbes [1]. Since the early discovery of the antibiotic penicillin in 1928 (produced by the mould Penicillium notatum), antibiotics have played a major role in protecting human health by tackling infectious pathogens [1-3].

However, the continuous use of antibiotics against microbes is coupled with the potential development of resistances as the target organisms can develop or acquire mechanisms to withstand the effect of the antibiotic agent [4-6]. The intensive and at times improper and uncontrolled use of antibiotics to treat minor infections (human and veterinary medication) in addition to the non-therapeutic use in the agricultural sector (for example as growth promoters in farm animals) have heightened the occurrence of microbial strains resistant to antibiotics [6-8]. At the same time this practice has led to the presence of antibiotics and antibiotic resistance genes in the environment [9, 10]. Consequently, antibiotic resistant strains can be transferred from the environment to humans. This might take place via faecal contaminated water, bacterially contaminated food materials such as meat or dairy products and also through contaminated produce fertilized by animal manure [1, 7]. This scenario has caused serious concerns as the development of new antibiotics with the ability to tackle resistant pathogenic strains has been somewhat slow, therewith intensifying the potential spread and development of resistant strains [1, 2, 5, 11]. Multidrug resistances have been reported for many well-known bacterial human pathogens such as Staphylococcus aureus, Mycobacterium tuberculosis or Pseudomonas aeruginosa [2, 5, 6], urging the necessity of discovering new classes of antibiotics [11].

The decline in discovering new antibiotics has been attributed to several factors, ranging from declined investments in pharmaceutical research, difficulties in discovering new classes of antimicrobial agents and the continuous discovery of analogues of already known classes [5].

Soil bound microorganisms such as moulds (Penicillium spp., Cephalosporium spp.) or actinomycetes (Streptomycyes spp.) have been the traditional source of antibiotics [4, 11]. Such organisms are typically isolated directly from soil samples by using non-selective artificial solid or liquid media [5], or by screening for antibiotic producing bacteria using agar overlay techniques or similar approaches routinely employed to detect antibiotic residues [12, 13]. These isolates are then tested as pure cultures for the production of bioactive compounds targeting bacterial pathogens [1, 2]. Albeit this approach was successful in leading to the discovery of potential antimicrobial candidate compounds in the past, this screening strategy has increasingly led to the continuous re-isolation of already known organisms (for example Streptomycyes spp.) and previously described antimicrobial compounds [2, 11]. At the same time traditional screening approaches are now regarded as labour intense and somewhat expensive, thus further adding to the slow progress in antibiotic discovery [1].

New antibiotics are urgently needed and therefore alternative screening approaches enabling the discovery of new organisms and new compounds are an option to overcome the dependency on commonly isolated soil actinomycetes such as Streptomycyes spp. [1, 2]. The screening of under explored environmental materials or ecological niches (sea mud, sea weeds, animal dung, tropical forests etc.) has already resulted in the discovery of novel microorganisms producing promising bioactive metabolites [2, 11].
Promising strategies include targeting novel microorganisms with unique metabolic functions, such as predatory bacteria which have been shown to not only produce bioactive metabolites but also to actively “hunt” for their prey [14]. Microbial predation typically involves the ability of organisms to search for their target organisms and then to establish cell to cell contact followed by the release of antibiotic compounds and/or secretion of hydrolytic enzymes which lyse the prey cells which therewith serve as substrate for growth [15-19]. Predatory delta Proteobacteria such as members of the orders Bdellovibrionales and Myxococcales have been found in diverse ecosystems with myxobacteria in particular being able to feed on a large variety of microscopic prey organisms such as Gram positive and Gram negative bacteria, fungi and even algae [16, 20-23].

Myxobacteria have been isolated from soil samples of all continents representing the different climate zones [15, 17, 24], from compost [15], fresh- and seawater samples [25], tree bark [26, 27], sea sand [25] and animal (mostly herbivore) dung [17, 22 28]. These organisms represent a unique prokaryotic life style as they are able to form multicellular fruiting bodies and hunt prey similar in style to a wolf pack [18, 23]. As myxobacteria are prolific producers of novel bioactive metabolites [29-31], they are a promising alternative when screening for new bioactive compounds [32, 33].

As mentioned above, animal dung has been established as an excellent source for the isolation of myxobacteria. In addition, herbivore dung extract is an ingredient recommended for isolation media targeting myxobacteria [28, 34]. This material is essentially composed of mixed organic matter and a large number of potential prey microorganisms. The screening of easily available herbivore dung such as that of Zebra as a potential source of bacteriolytic prokaryotes can therefore be done by employing a simple but specific approach using microbial bait as sole source of carbon and energy as reported previously [15, 24]. However, so far it appears that the use of dung for the isolation of myxobacteria has not included indigenous South African herbivores such as Zebra or Giraffe while the use of droppings from herbivores such as rabbit, hare or deer was reported [21, 28, 35]. Using dung from less familiar herbivores might therefore increase the chance of obtaining producers of novel secondary metabolites [36].

The aim of this study was therefore to verify the baiting technique as a way to isolate bacteriolytic myxobacteria from previously untapped herbivore droppings.

2. Materials and methods

2.1. Sample collection
Zebra droppings used in this study were freshly collected from the Bisley Nature Reserve in Pietermaritzburg (KwaZulu-Natal). Samples were placed in sterile bottles and immediately transported on ice to the laboratory. Samples were stored in a refrigerator at 6°C and processed further within 1 week.

2.2. Enrichment procedure
The enrichment and isolation of predatory myxobacteria was conducted using a baiting technique as previously described [30] with the following modifications. *Escherichia coli* (ATCC 8739) cells were grown in LB broth (Merck) for 48 hours on a shaker (150 rpm) at 25°C, thereafter centrifuged at 12000 x g for 15 minutes and washed 3 times with sterile saline (0.85% sodium chloride in distilled water). The cell pellet was suspended in sterile saline and adjusted to a cell concentration of about 10^13 cells/ml using a bacterial counting chamber. Thereafter the cell suspension was heat inactivated by mixing an appropriate volume thereof with hot (80°C) sterile water agar (15g bacteriological agar (Merck) in 1 litre distilled water) which was then allowed to cool to 55°C. Either filter sterilized cycloheximide (Merck) or filter sterilized nystatin (Merck) solution was then added to a final concentration of 100 μg/ml or 20 μg/ml respectively to minimize growth of opportunistic coprophilic fungi. Small samples of Zebra dung (about 2 x 3 cm) were embedded into the water agar containing heat inactivated *E. coli* (10^10 per ml agar) as bait. These plates were incubated in a moist chamber at room temperature (25°C) and regularly examined for swarms and fruiting bodies for up to 10 weeks.

2.3. Isolation procedure
Using a sterile inoculation loop, samples of visible fruiting bodies - verified as such by use of a stereomicroscope and an inverted light microscope - were streaked onto water agar spiked with 10^10 cells of *E. coli* per ml. In this case the water agar contained cycloheximide (100 μg/ml) and penicillin G (25 μg/ml) to control fast growing fungi and Gram positive bacteria.

2.4. Cultivation of isolates
One orange coloured myxobacterial isolate - designated strain NSM1 - was cultivated at 30°C on water agar containing *E. coli* as bait as specified above (2.3.). Plates were checked for the presence of visible growth after 2 weeks.
2.5. Microscopy of the isolate NSM1

Fruiting bodies were examined routinely using a stereomicroscope while vegetative cells of the isolate were examined using wet mounts or Gram stained preparations using brightfield and phase-contrast microscopy. Gliding motility on agar plates was checked by use of an inverted light microscope. The presence of different developmental stages of the isolate was analysed by environmental scanning electron microscopy (ESEM). The isolate was grown as above (2.4) at 30°C for four days and a block of agar containing orange coloured vegetative cells and fruiting bodies was cut out with a sterile dissecting knife and mounted onto a stub. A Philips XL130 ESEM with Lab6 gun was used at optimally adjusted vacuum, temperature and humidity levels.

2.6. Biochemical characterization of the isolate NSM1

Catalase activity was detected by using 3% hydrogen peroxide solution, the oxidase test was done according to Kovacs [37] while urease was determined by inoculating filter sterilized urease broth (Merck) followed by 4 days incubation at 30°C. Cellulose utilization was determined using CMC (carboxymethylcellulose) agar plates [38] being flooded after 4 days with Congo red solution (0.01%) to detect CMC hydrolysis. Amylase was determined on nutrient agar plates containing 0.2% soluble starch by flooding plates after 4 days incubation with Lugol’s iodine solution. The ability to liquefy gelatine and to hydrolyse casein was assessed as previously reported [39]. Appropriate bacterial strains were employed as positive and negative controls for the biochemical tests.

2.7. Analysis of the 16S rRNA gene sequence of isolate NSM1

A single colony of isolate NSM1 was harvested from water agar spiked with *Saccharomyces cerevisiae* and suspended in 100 μl sterile TrisHCl buffer (50 mM, pH 7.4) and treated by six freeze-thaw cycles consisting of 2 min. in liquid nitrogen and 2 min. at 95°C. A 1 μl sample was used for target gene amplification by PCR using the previously described primers fD1 (5’-AGAGTTTGATCCTGGCTCAG-3’, positions 7 to 26 in the *Escherichia coli* 16S rRNA gene) and rP2 (5’-ACGGCTACCTTGTTACGACTT-3’, positions 1513 to 1494 in the *Escherichia coli* 16S rRNA gene) [40]. The amplification product (1368 bp) was sequenced (Inqaba Biotec, Pretoria, South Africa) and compared to 16S rRNA gene sequences deposited in GenBank ([http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). A phylogenetic tree using sequences for selected species within the order Myxococcales (delta Proteobacteria) deposited within RDP (ribosomal database project, rdp.cme.msu.edu) was generated, based on a sequence alignment established with clustal w and the neighbour joining method using MEGA 5.21 [41].

2.8. Evaluation of lytic activity

Six microbial species representing both Gram positive (*Mycobacterium smegmatis* (ATCC 607), *Staphylococcus aureus* (ATCC6538)) and Gram negative bacteria (*Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella Typhimurium* (ATCC 14028)) and a eukaryotic microorganism (*Saccharomyces cerevisiae* (DSM1333)) were used. Prey microorganisms were grown in LB broth (Merck) for 48 hours at 25°C at 150 rpm, cells were then harvested by centrifugation at 12000 x g for 15 minutes, and the supernatant was discarded. For the demonstration of lysis of bait cells, cells were suspended in sterile saline (0.85% sodium chloride in distilled water) and adjusted to an appropriate cell density (typically 10^7-10^10 cells/ml) using a suitable counting chamber. An appropriate quantity of cell suspension was then added to hot sterile water agar (inactivated prey) or, alternatively, by adding an appropriate aliquot to sterile water agar cooled down to about 50°C (active prey). The myxobacterial isolate was then streaked onto water agar containing bait with plates being examined after 4 days for the presence of zones of clearance. As water agar contains no carbon source, prey cells were the only substrate available for myxobacterial growth.

3. Results and discussion

3.1. Characterization of the isolate NSM1 and its life cycle stages

Using the baiting technique with inactivated *E. coli* cells as prey and Zebra dung as inoculum, orange fruiting bodies of about 100 μm in diameter became visible on the surface of the Zebra droppings after eight weeks of incubation at 25°C in a moist chamber (Figure 1A). The appearance of these structures was soft and mucous which is typical for members of the genus *Myxococcus* [24]. The ability to develop multicellular fruiting bodies when experiencing nutrient depletion is a feature typical for the life cycle of myxobacteria [32, 42]. However, shape, size and colouration of fruiting bodies vary among different myxobacterial species [22, 24, 28, 43] while myxobacteria unable to form such fruiting bodies have been reported [44]. In addition to fruiting bodies, myxobacteria typically show swarming behaviour whereby the cells actively move across surfaces into unoccupied areas [24, 42]. Thus the observed swarming of vegetative cells on inoculated water agar plates containing *E. coli* cells as bait was not unexpected on microbiological grounds (Figure 1B). Myxobacterial swarming is a strategy for cooperative feeding, as myxobacteria grow while they actively spread over a solid surface containing nutrients such as prey which they can utilize [42]. To further evaluate the structure and
development of fruiting bodies of the isolate NSM1, environmental scanning electron microscopy was employed. The ESEM analysis revealed the different developmental stages of fruiting body formation within the life cycle of the isolated strain. Thus early cell aggregates and assemblages as well as premature (about 70 µm in diameter) and mature fruiting bodies of about 100 µm in diameter (mature stage) were observed (Figure 2).

![Image of fruiting bodies](image1.png)

**Fig. 1** A. Formation of soft and mucous orange pigmented fruiting bodies on the surface of Zebra dung embedded into water agar containing *E. coli* as bait after 8 weeks incubation in a moist chamber at 25°C. Scale bar = 500 µm. B. Visible swarming of myxobacteria from Zebra dung onto water agar containing *E. coli* as bait.

![Image of different stages](image2.png)

**Fig. 2** Different stages of fruiting body development observed (ESEM) in isolate NSM1. A. Scale bar = 2 µm. B. Scale bar = 2 µm C. Scale bar = 10 µm D. Scale bar = 10 µm.

Yamanaka *et al.* [27] reported that the size of fruiting bodies of myxobacterial isolates ranged from 60-600 µm, while Shimkets *et al.* [22] specified that the fruiting body size - depending on the species - might fall within a range of 10-1000 µm. Thus the diameter for the fruiting bodies observed for isolate NSM1 is within the expected range. The above mentioned orange fruiting bodies found growing on Zebra dung produced, as expected, myxospores. These ellipsoidal myxospores measured about 1.8 x 1 µm. The rod shaped and slender vegetative cells of the Gram negative isolate measured about 3 x 1 µm length. The isolate was oxidase and catalase positive, indicating besides an aerobic life style...
the use of aerobic respiration. Most myxobacterial species are found inhabiting aerobic habitats such as upper layers of soil and animal dung surfaces [17], although an anaerobic myxobacterial species, *Anaeromyxobacter dehalogenans*, has been reported [45]. In addition, the isolate NSM1 exhibited urease and amylase activities which are features previously reported for species within the genus *Myxococcus* [27].

![Phylogenetic affiliation](image)

**Fig. 3** Phylogenetic affiliation of the isolated strain NSM1 (black circle) based on the comparison of its 16S rRNA gene sequence with a number of selected 16S rRNA gene sequences for myxobacterial type strains (open circles). The scale bar represents 2 estimated changes per 100 nucleotides. *Escherichia coli* was used as out-group (black diamond).

The sequence analysis of the 16S rRNA gene of strain NSM1 revealed high similarity (99%) to the 16S rRNA gene sequence of the type strain *Myxococcus fulvus* (ATCC 25199; NCBI reference sequence NR_043946.1) and more than 99% similarity to the 16S rRNA gene sequence of an environmental isolate of *Myxococcus fulvus* ([35], GenBank: GU477561.1), thereby confirming that this isolate is a member of the genus *Myxococcus* and making its assignment to the species *Myxococcus fulvus* likely. Phylogenetic analysis demonstrated a close relationship between the isolated strain NSM1 and *Myxococcus* spp. (Figure 3) and confirmed the overall tree topology reported recently for the *Myxococcales* [46]. However, although the ability to utilize starch was reported for *Myxococcus fulvus* [27], a more recent study found that 11 strains representing different species within the genus *Myxococcus* were unable to
decompose starch [47]. In addition, the isolate NSM1 hydrolysed cellulose and appeared to form a stalk carrying the fruiting bodies (Figure 2D), which are not features of *Myxococcus fulvus* [27].

### 3.2. Evaluation of the ability to utilize bait cells for growth

The isolate NSM1 was able to degrade and utilize the cells of both Gram positive and Gram negative bacteria. In addition, yeast cells were used as sole source of carbon and energy. Upon incubation of the isolate on water agar in the presence of prey cells as sole source of carbon and energy, clear zones of lysis were observed (Figure 4). According to Singh [15], *Myxococcus fulvus* can hydrolyse both Gram negative and Gram positive bacteria. However, this species shows an apparent preference for Gram negative bacteria [16]. This preference for Gram negative over Gram positive bacterial prey was recently confirmed for strains of *Myxococcus xanthus*, *Myxococcus flavescens*, *Myxococcus virescens* and *Myxococcus macrosporus* [23]. Strains representing Gram negative genera such as *Salmonella*, *Vibrio*, *Proteus*, *Escherichia*, *Serratia* and *Shigella* were lysed by *Myxococcus fulvus* [20, 48]. Similarly, the isolate NSM1 lysed *Escherichia coli*, *Pseudomonas aeruginosa* and *Salmonella Typhimurium*. The same authors [20, 48] found that prey cells from Gram positive genera such as *Staphylococcus*, *Lactobacillus*, *Micrococcus* and *Bacillus* were lysed by *Myxococcus fulvus*. However, *Mycobacterium phlei* was not lysed by *Myxococcus fulvus* [20]. Interestingly, isolate NSM1 was able to lyse both *Staphylococcus aureus* and *Mycobacterium smegmatis*. The principal ability to degrade protein was demonstrated for isolate NSM1 by its ability to hydrolyse gelatine and casein, a feature reported for other *Myxococcus* species [21, 27].

**Fig. 4** Bacteriolytic activity of isolate NSM1 against *Salmonella Typhimurium* bait cells in water agar after incubation at 30°C for five days (plate on the left). On the right the negative control shows that *E. coli* is not able to lyse *Salmonella Typhimurium* bait cells.

In fact myxobacteria hydrolyse proteins [22] and nucleic acids to obtain essential amino acids and phosphate for growth [42]. The observed zones of clearance (Figure 4) indicate the ability of the isolate NSM1 to feed on cells as substrate which would require the ability of the cells to hydrolyse bacterial proteins and possibly lipids and carbohydrates.

### 4. Conclusion

The use of a simple baiting technique showed that the droppings of indigenous South African herbivores such as Zebra are a potential source for the isolation of predatory myxobacteria. Such isolates might in turn be valuable sources for new bioactive secondary metabolites exhibiting antibiotic activity.

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**References**


