Journal of Science Research

Subsistence of four bacteria isolated from Nigerian aquaculture farms on oxytetracycline

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Abstract

The ability of 147 oxytetracycline (OTC)-resistant bacteria isolated from aquaculture ponds in Ibadan, Nigeria, to subsist on OTC as a source of nutrient for growth was investigated in this study. Four bacteria identified by 16S rDNA sequencing as *Bacillus pumilus* Aa8, *Bacillus pumilus* Ab11, *Stenotrophomonas maltophilia* Ng7 and *Klebsiella pneumoniae* Nf6 utilised 4.8, 13.1, 16.5 and 19.0 % of the OTC supplied in the growth medium in 72 hours with degradation rates of 8.0, 21.8, 27.5 and 31.7 µg/day respectively. The results showed that bacteria capable of subsisting on tetracycline (TET) antibiotics are present in the environment. However, the risk of possible transfer of resistance determinants to other environmental bacteria by these strains is a disincentive to their use in the clean-up of antibiotic contaminated ecosystems.

Keywords: Antibiotic resistant bacteria; *Bacillus pumilus; Klebsiella pneumoniae;* Nigerian aquaculture; *Stenotrophomonas maltophilia.*

Introduction

Environmental contamination by antibiotics is an emerging global environmental problem [1, 2, 3]. The large scale production and use of antibiotics in human and veterinary medicine, agriculture, aquaculture, and horticulture have led to the release of significant quantities of antibiotics into the environment with serious consequences [4-7]. The presence of antibiotics in the environment may lead to the development of antibiotic resistance in environmental bacteria species with possible dissemination of the resistance determinants to human pathogenic bacteria [8-10]. In addition, antibiotics may alter the composition and diversity of microbial flora of natural ecosystems thereby upsetting critical ecosystem functions such as nutrient cycling, decomposition and energy flow [10-12]. This makes the development of an eco-friendly method for the clean-up of antibiotic contaminated ecosystems an urgent task.

Owing to their broad spectrum of activity, low cost and low toxicity, tetracyclines (TET) are used widely in the treatment of a number of human infections [13]. It is also used widely as growth promoter in animal production, for prophylaxis in plant agriculture and in aquaculture [14-16]. Consequently, it is one of the classes of antibiotics most commonly detected in the environment [17]. Oxytetracycline (OTC), a member of the TET family of antibiotics, is particularly detected widely in surface waters and soil with concentrations up to 2 mg/l [18]. Further, because they are easily adsorbed to sludge and humic substances in sediments [19], TET are usually reported as non-biodegradable compounds raising fears about their possible persistence in the aquatic ecosystem.

Natural ecosystems are home to a large diversity of micro-organisms that play important roles in ecosystem functions both as individuals and as a community. These micro-organisms degrade a vast array of organic compounds including antibiotics as part of their metabolism and general survival [20]. Thus, they play important roles in determining the fate of pollutants (including antibiotics) in nature through various forms of modifications [21]. Ability to resist the toxicity of pollutants is an important first step in

biodegradation by micro-organisms. This is perhaps one of the reasons why the utilisation of anti-microbial substances by bacteria as source of nutrient for growth is currently attracting global research attention as a means of cleaning antibiotic contaminated ecosystems. It is thought that catabolism of antibiotics may be one of the steps in the evolution of antibiotic resistance [22]. Growth-related catabolism is also expected to play an important role in the degradation of contaminating antibiotics and their eventual removal from antibiotics polluted ecosystems, strongly suggesting a role for antibiotic-resistant bacteria in this process. However, opinion is still divided as to whether antibiotic-resistant bacteria actually go beyond the resistance trait to subsist on antibiotics [23]. Also, the public health implication(s) of antibiotic degradation by antibiotic-resistant bacteria is yet to be fully understood by the scientific community, creating a strong need for continual investigation of this important phenomenon. There is also a strong and compelling need to investigate the fate of antibiotics in the tropical ecosystem such as Nigeria, where there is little or no information on the behaviour of antibiotics which enters the environment through untreated wastewater from domestic, industrial, agricultural and aquaculture sources.

Early evidence that bacteria can use antibiotics as a source of nutrient for growth included the work of Abd El-Malek *et al* [24], Fenton *et al* [25] and Johnsen [26] who reported species of *Streptomyces* and *Pseudomonas* capable of utilising chloramphenicol, streptomycin and benzylpenicillin as carbon and nitrogen sources respectively. Recently Dantas *et al* [22] reported that bacteria capable of subsisting on antibiotics as a source of nutrient for growth are abundant in the soil ecosystem. Barnhill *et al* [27] further investigated the phenomenon among 572 animal isolates of *Salmonella*. All the 12 antibiotics tested except TET enabled the subsistence of at least one of the tested *Salmonella* strains. Xin *et al* [10] also reported the subsistence of four *Klebsiella pnuemoniae* and one *Escherichia fergusonii* isolated from human intestine on chloramphenicol. Similarly, Zhang and Dick [21] reported that penicillin and neomycin enabled the subsistence of 19 soil bacteria not previously exposed to penicillin and neomycin.

Recently, several studies also reported the utilisation of antibiotics belonging to different classes as sources of nutrient for growth by bacteria species. These include the degradation of various sulphonamides by *Microbacterium lacus* strain SDZm4 [28], species of *Pseudomonas, Brevundimonas, Variovorax* and *Microbaterium* [2], *Microbacterium* species [29, 30]. Gao *et al* [31] also reported the degradation of erythromycin by *Pseudomonas* sp. ERY-E and used same to treat surface water contaminated with low concentrations of erythromycin in a biological aerated filter.

However, reports of bacteria degrading TET in pure axenic cultures are not very common. Most of the previous study of TET degradation has hitherto focused on its degradation in different environmental matrices or test systems via abiotic and/or biotic processes. While a few of the studies have attributed observed dissipation of TET to microbial action [32, 33], and two previous studies have indeed reported the degradation of TET by the yeasts *Xylaria digitata* [34] and *Trichosporon mycotoxinivorans* XPY-10 [35], very few studies have reported the degradation of TET by bacteria growing in axenic cultures.

This study therefore investigates the ability of OTC-resistant bacteria isolated from fish farms in Ibadan, south-western Nigeria to subsist on OTC as a source of nutrient for growth. Our assumption is that bacteria capable of degrading TET in axenic cultures exist in the natural ecosystem and that it should be possible to find such bacteria in ecosystems exposed to TET as well as in those without any history of such previous exposure. To test these hypotheses, we isolated OTC-resistant bacteria from four ponds where OTC is regularly used in fish health management and six ponds without history of such previous exposure and tested them for subsistence on OTC. To the best of our knowledge, this is the first study reporting the utilisation of oxytetracycline, an antibiotic widely used in fish production, as a source of nutrient for growth by bacteria from tropical aquaculture environment.

Materials and methods

Materials

OTC hydrochloride ($C_{22}H_{24}N_2O_9$.HCl, CAS 2058-46-0, Mol. Wt. 496.89g, $\geq$95% (HPLC purity) was obtained from Sigma-Aldrich (St Louis MO). Pond water was collected from 10 fish ponds in Ibadan, south-western Nigeria in March 2013. The fish-farms have an average age of 5 years and OTC has been used in four of the ponds for more than three years before the time of this study primarily as additive to feed and pond water. The water samples were transported to the laboratory on ice for isolation of bacteria within 6 hours of collection.

Bacterial isolation

The total OTC-resistant bacteria (TOBC) of the pond water samples were estimated by plating aliquots of appropriate dilutions of the pond water on Mueller Hinton Agar (MHA) (Oxoid) supplemented with 20

µg/ml of OTC as described by Gosh and LaPara [36]. Plates were incubated at 30°C for 48 hours and colonies growing on the OTC supplemented agar plates were counted and used to estimate the TOBC. Distinct colonies of bacteria growing on the plates were selected and purified by streaking on fresh plates before storage in glycerol broth (15 %) at -15°C.

Screening for subsistence on OTC

The OTC-resistant bacteria were screened for ability to subsist on OTC as a source of nutrient for growth as described by Barnhill *et a*l [27]. Briefly, single carbon source (SCS) agar was prepared as previously described [22] and the pH adjusted to 7.0. The medium was supplemented with 0.5 mg/l of filter sterilised OTC (0.2 µm pore size, Nucleopore, Pleasanton CA) as source of carbon and energy for growth to form single carbon source-OTC (SCS-OTC) agar. A loopful (10 µl) of standardised saline suspension (0.5 MacFarland Standard) of overnight cultures of each isolate was streaked on the SCS-OTC agar plates. The plates were incubated in the dark at 30°C for 48 hours and isolates growing on the plates with $\geq$10 colonies were selected as potentially capable of subsisting on OTC as a source of nutrient for growth [27].

Growth on OTC in liquid medium

The growth of isolates potentially capable of subsisting on OTC was monitored by measuring the optical density at 600 nm (OD_{600}) in liquid SCS-OTC medium at 0, 24, 36, 48, 60 and 72 hours. Aqueous stock solution of the OTC was filtered separately using a Millipore Syringe filter (0.2µm pore size, Nucleopore, Pleasanton CA) before addition to autoclaved SCS medium to a final concentration of 0.5 mg/l. Saline suspensions (0.5 ml) prepared from 3-4 identical colonies of overnight culture of the test bacteria on MHA (Oxoid) were standardised (0.5 MacFarland Standard) and used to inoculate the medium to a final volume of 100 ml. The experiment was conducted in amber coloured glass bottles wrapped with black polythene sheets to prevent photodegradation of OTC during incubation at 30°C. SCS-OTC medium without bacterial inoculation served as controls.

HPLC analysis

The quantities of OTC utilised as growth substrate by the test-bacteria after 72 hours of growth in liquid SCS-OTC medium was determined by HPLC. The growth media were centrifuged to remove cells and 2 drops of 0.1 M HCl added to prevent hydrolysis of residual OTC before HPLC analysis. Concentration of residual OTC in the growth medium were determined using a Water Alliance 2489/2695 system fitted with a UV-Visible detector set at 360 nm and an Agilent SB (250 mm x 4.6 mm, 5µm) C18 column. The column temperature was set at 30°C and the injection volume was 10 µl at a flow rate of 1.4 µl/ml. The mobile phase consisted of 0.01M Oxalic acid (650 ml) and 350 ml of Methanol acidified with 4 drops of Perchloric acid (Solution A) and Methanol (Solution B). The mobile phase was Solution A: B 60:40 (v/v) with a gradient (30 min) to Solution A: B 40:60 (v/v) at room temperature. A standard curve was previously plotted with 0.25, 0.5, 1.0, 2.0 and 5.0 mg/l of OTC.

16S rRNA identification

The 16S rRNA gene of the test-bacteria were amplified with universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 1492R (GTT TAC CTT GTT ACG ACT T-3') to produce amplicons of about 1500 bp. Amplification was performed at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 45 seconds, extension at 72°C for 1 minute and a final extension at 72°C for 10 minutes. The PCR products were cleaned and sequenced (Iquaba Biotech, South Africa) and the sequences were used to perform a BLAST search in the GenBank database to confirm the identity of the isolates.

Results

Bacterial isolates and screening for growth on OTC

The TOBC of the ponds ranged from 1.95 to 3.87 $\times 10^8$ cfu/ml. The population of OTC-resistant bacteria in the four ponds exposed to OTC (A: 3.87×10^8 cfu/ml, B: 3.40×10^8 cfu/ml, C: 2.95×10^8 cfu/ml, D: 3.75×10^8 cfu/ml) were higher than in the six ponds where OTC was not used (E: 1.95×10^8 cfu/ml, F: 2.10×10^8 cfu/ml, G: 2.80×10^8 cfu/ml, H: 1.98×10^8 cfu/ml, I: 2.60×10^8 cfu/ml, J: 2.72×10^8 cfu/ml). A total of 147 distinct colonies were selected from OTC (20 µg/ml) supplemented MHA plates. The isolates were considered as OTC-resistant using the Clinical and Laboratory Standards Institute (CLSI) interpretive standard for tetracyclines which was set at $\geq$16 µg/ml for *Enterobacteriaceae, Acinetobacter* and other non-enterobacteriaceae [37]. Further screening on SCS-OTC agar reduced the number of isolates to 37 potentially capable of subsisting on OTC as a source of nutrient for growth. Fourteen (37.8 %) of these bacteria were isolated from ponds without history of OTC use while the remaining 23 (62.2 %) were from ponds exposed to OTC.

The growth of the 37 bacteria isolates in SCS-OTC broth was monitored using UV-Visible spectrophotometry to confirm their ability to subsist on OTC as source of nutrient for growth. Four isolates

(10.8 %) showed evidence of growth on OTC, manifested as increase in OD_{600} within the experimental period of 72 hours (Figure 1). Two of the isolates coded Ng7 and Nf6 were isolated from ponds without history of OTC exposure while the other two, coded Ab11 and Aa8 were from ponds with history of OTC usage. The remaining isolates (*n*=33) showed little or no growth in liquid SCS-OTC medium throughout the experimental period. Most were also growing as very tiny colonies on plates during screening for subsistence on SCS-OTC agar plates. 16S rRNA sequencing showed that the isolates shared 98-99 % identity with strains in the GenBank. Isolates Ab11 and Aa8 shared 98 and 99% sequence identities respectively with *Bacillus pumilus* strains HT-Z41 and JBS-32 with GenBank accession numbers KJ526890.1 and KM675994.1. While isolates Nf6 and Ng7 shared 98 and 99 % identities respectively with *Klebsiella pneumoniae* 32192 (accession no. CP010361.1) and *Stenotrophomonas maltophilia* LH15 (accession no. KM893074.1) (Table 1).

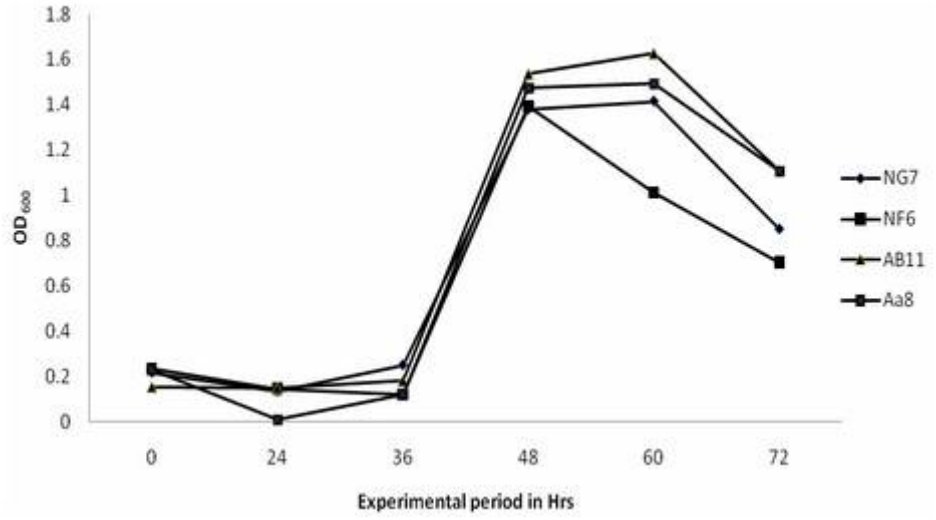

Figure 1. Growth of the test bacteria in SCS-OTC medium.

Table 1. Phylogenetic identities and OTC degradation (µg/day) by the OTC-resistant bacteria isolated from pond water in Ibadan.

Isolates code	16S rRNA gene sequence similarity (%)	*Closest similarities in the GenBank	#Concentration of OTC degraded (µg)	Rate of OTC degradation (µg/day)
Aa8	99	*Bacillus pumilus* JBS-32 (KM675994.1)	24.0 (4.8 %)	8.0
Ng7	99	*Stenotrophomonas maltophilia* LH15 (KM893074.1)	65.5 (13.1 %)	21.8
Ab11	98	*Bacillus pumilus* HT-Z41 (KJ526890.1)	82.5 (27.5 %)	27.5
Nf6	98	*Klebsiella pneumoniae 32192* (CP010361.1)	95.0 (19.0 %)	31.7

*Accession number in parenthesis; #Concentration of OTC degraded relative to that of uninoculated controls.

Subsistence on OTC

The four-bacteria isolates showed a long lag phase which lasted for about 24 hours followed by a period of slow growth between 24 and 36 hours. This was followed by a rapid increase in OD_{600} between 36 and 60 hrs before a drop indicating cessation of growth (Figure 1). OD_{600} for isolates Ng7, Ab11 and Aa8 rose from initial values of 0.22, 0.153 and 0.228 at 0 hour to 1.415, 1.628 and 1.492 respectively at 60 hours before a decrease between 60 and 72 hours. In contrast, the growth of Nf6 peaked at 48 hours when OD_{600} rose to 1.395 before dropping at 60 hrs (Figure 1).

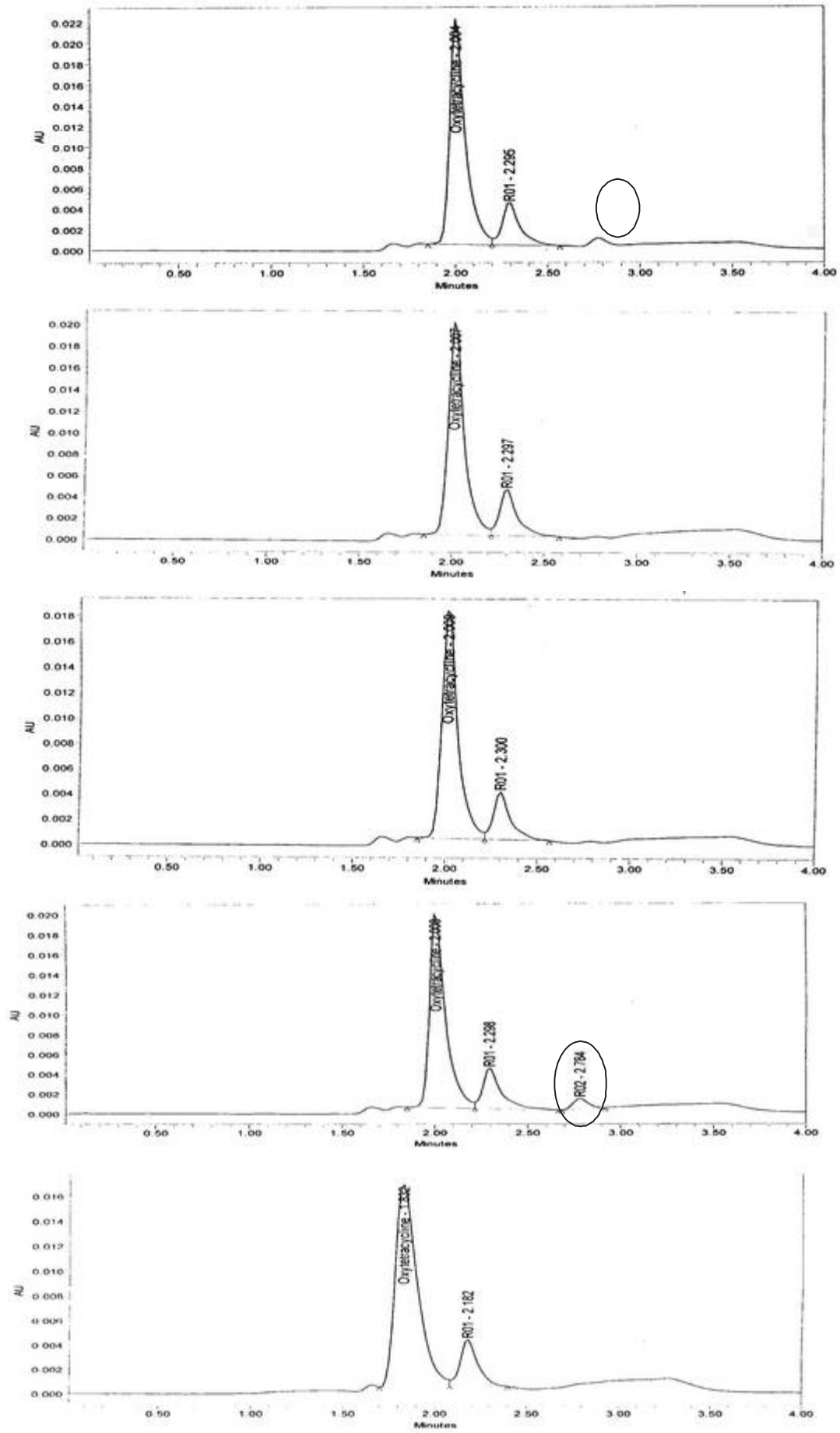

Figure 2. HPLC chromatograms of the cell-free culture supernatants of *Bacillus pumilus* A8, *B. pumilus* Ab11, *K. pneumoniae* Nf6, and *S. maltophilia* Ng7 showing suspected degradation products of oxytetracycline (red rings).

Quantitative HPLC analysis showed that the isolates reduced the concentration of OTC supplied in the growth medium by as much as 4.8 to 19.0% within the 72 hours period of the experiment. This is evidenced by reductions in the peak areas of the experimental cultures compared to that of the uninoculated control (Figure 2). *B. pumilus* Aa8 showed the least OTC degradation among the four bacterial isolates. The bacteria degraded 24.0 µg (4.8 %) of the OTC supplied in the growth medium with a degradation rate of 8.0 µg/day. *S. maltophilia* Ng7 and *B. pumilus* Ab11 degraded 65.5 µg (13.1 %) and 82.5 µg (16.5 %) of OTC with degradation rates of 21.8 µg/day and 27.5 µg/day respectively, while *K. pneumoniae* Nf6 degraded the highest quantity, 95.0 µg of OTC (19.0 %) with a degradation rate of 31.7 % (Table 1). Additional peaks suspected to be degradation products of OTC were identified in the chromatograms of *B. pumilus* Aa8 and *S. maltophilia* Ng7. These peaks were not present in the chromatogram of the control (Figure 2).

Discussion

This study focused on the isolation of pure cultures of OTC-resistant bacteria capable of metabolising OTC, a tetracycline antibiotic, from Nigerian aquaculture farms and its utilisation as nutrient source for growth in mineral medium containing OTC as the sole carbon source. Previous studies on degradation of tetracycline published so far, mainly referred to the dissipation of the compounds in different environmental matrices including animal manure [9], soil [38], river-water and sediment [32] and marine fish farm sediments [39]. Reports of tetracycline degradation by pure cultures have so far been limited to yeasts [34, 35]. The focus on pure cultures of bacteria capable of subsisting on OTC is important for some reasons. First, it will enable a linking of OTC utilisation with specific bacterial species which can be applied as functional strains in treating OTC-contaminated wastewater. Secondly, in view of the potential for the transfer of resistance determinants by antibiotic resistant bacteria species, it will also enable an assessment of the public health risks that may be associated with the application of such strains in wastewater treatment.

Four bacteria out of 147 OTC-resistant bacteria screened were able to utilise OTC for growth, indicating that while OTC resistance is common among isolates of the present study, subsistence on OTC is not a common phenomenon. Similar to this observation, Ooishi and Tosa [18] also reported in a recent study of the occurrence of tetracycline- resistant and tetracycline-degrading bacteria in wastewater treatment effluent and environmental water systems that not all bacteria species in tetracycline spiked river water were capable of degrading tetracycline. Indeed, preliminary screening on SCS-OTC agar reduced the number of OTC-resistant bacteria (147) initially isolated on MHA plates supplemented with 20 µg/ml OTC to 37 bacteria potentially able to utilise OTC as nutrient source for growth. However, these were further reduced to four when growth of these strains was evaluated in SCS-OTC broth. Thus, it is quite possible that the resistant-bacteria might have lowered their metabolic functions to survive rather than grow in the medium with OTC as a source of nutrient [13] thus leading to little or no change in cell number as obtained in this study. It is also possible that while the isolates were able to resist the toxicity of OTC in nutrient rich MHA supplemented with OTC used for their isolation, the combined toxicity of OTC and organic nutrient starvation was not favourable for their growth resulting in tiny colony sizes on SCS-OTC agar.

Sequencing of the 16S rDNA identified the four OTC subsisting bacteria as species of *Bacillus* (2 No), *S. maltophilia* and *K. pneumoniae.* Members of these bacteria genera are involved in the degradation of several environmental pollutants including antibiotics. *Bacillus* spp., *S. maltophilia* and *K. pneumoniae* in particular are increasingly being mentioned in recent reports among bacteria capable of subsisting on various antibiotics as sources of nutrients for growth [10, 21, 40]. However, considering the important roles played by these bacteria genera in human infections [41, 42], the increasing report of their subsistence on various antibiotics as a source of nutrient for growth is a disturbing observation. The far-reaching public heath implication of this is that through metabolism of antibiotics, they can protect themselves as well as other pathogenic bacteria from the toxic action of respective antibiotics [43] and also disseminate resistance determinants to the protected species.

The concentrations of OTC (4-19 %) removed within three days in the nutrient poor SCS-OTC medium used in the present study by the isolated bacteria are lower compared to concentrations of OTC (31-58 %) removed by eight bacteria in Meuller Hinton broth supplemented with OTC in 21 days reported by Maki *et al* [39]. The lower removal efficiency notwithstanding, utilisation of OTC under poor nutrient conditions by these bacteria strains proved their ability to utilise OTC as sole source of carbon and energy and may be potentially useful in designing biotechnological processes for the remediation of antibiotic contaminated wastewater. It also indicted that nutrients augmentation may possibly improve OTC utilisation by the strains due to cometabolism or diauxic effects. Cometabolism and diauxic processes are

increasingly being reported as important processes in the degradation/transformation of antibiotics by bacteria species [2, 44-46].

In summary, this study has led to the isolation and identification of bacteria that were capable of subsisting on OTC from ecosystems exposed to OTC as well as those without history of OTC exposure as obtained in similar studies [10, 21, 22, 27, 47] . *B. pumilus* Aa8, *B. pumilus* Ab11, *S. maltophilia* Ng7 and *K. pneumoniae* Nf6 subsisted on OTC contrary to earlier report that antibiotics of the TET class did not enable the subsistence of *Salmonella* [27]. To the best of our knowledge, this is the first report of subsistence on antibiotic by bacteria from Nigerian aquaculture farms. This finding is significant in at least in two respects:

First, it has extended what is known of bacteria subsistence on antibiotics by identifying bacteria capable of subsisting on an antibiotic of the TET family. Furthermore, results of this study also provided insight into the ability of environmental bacteria to subsist on antibiotic and their potentials in degrading contaminating antibiotics in tropical aquatic ecosystems.

Second, the study also identified individual bacteria species capable of utilising OTC, a member of the TET family as a source of nutrient for growth in axenic cultures.

These findings have important implications for public health and the environment. Viewed from an environmental perspective, this trait may be useful in the removal of contaminating antibiotics in antibiotic polluted ecosystems. However, the possible transfer of virulence and antibiotic resistance determinants by these isolates to other bacteria species and their roles as important opportunistic pathogens are important factors that should be taken into consideration before their use in bioremediation of OTC contaminated ecosystems.

Acknowledgments

We are immensely grateful to the owners of the fish farms for their cooperation in sample collection.

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Journal of Science Research
Volume 14, 2015, ISSN 1119 7333
Citation: Adelowo, O. O., Lawal, O. E., and Adekanmbi, A. O. Subsistence of four bacteria isolated from Nigerian aquaculture farms on oxytetracycline.

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