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Metal-resistance encoding gene-fingerprints in some bacteria isolated from wastewaters of selected printerries in Ibadan, South-western Nigeria

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ABSTRACT

Several studies have reported the occurrence of metal-resistant bacteria and their genes in different wastewater, but there is a dearth of information on wastewater generated from printing operations as a probable source. This study aimed at fingerprinting metal-resistance encoding genes in bacteria recovered from wastewaters of selected printerries in Ibadan, Nigeria. Wastewaters from 10 selected printerries in Ibadan were collected monthly for 12 months. The metal composition of wastewater was determined using Atomic Absorption Spectrophotometry. Metal-resistant bacteria were isolated on metal-supplemented nutrient medium, and characterized using 16S rRNA gene sequencing. Metal-resistance genes were detected using specific primers and the presence of plasmids was determined using alkaline-lysis method. Forty metal-resistant bacteria belonging to six genera; *Bacillus*, *Klebsiella*, *Pseudomonas*, *Citrobacter*, *Providencia* and *Proteus* were identified. *cusCBA*, encoding resistance to copper and silver was detected in nine bacteria, while *pbrA* (encoding lead resistance) was detected in seven *Pseudomonas aeruginosa* isolates. *chrA*, encoding resistance to chromate ions, was detected in *Proteus mirabilis* PW3a and two isolates of *Pseudomonas aeruginosa*, while *chrB* was detected in *Providencia vermicola* PWAP₃ and *Proteus mirabilis* PW4c. *Bacillus stratosphericus* PW1b possessed the copper-resistance genes, *pcoA* and *pcoR*. Thirty-six bacteria (90%) of the total bacteria possessed plasmids larger than 10 Kb in size. In conclusion, wastewater generated from printing operations could be a potential source of metal-resistant bacteria and their genes.

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1. Introduction

Printing and the processes associated with it occupy a pivotal position in everyday life as no day is complete without human beings having a direct or indirect contact with various forms of printed items. These items include packaged consumer products, books, newspapers, journal articles, computer prints, photocopies and a host of other products. Inks, the organic or inorganic pigments employed in printing process contain coloured and colourless pigment particles dispersed in suitable solvents [1]. Modern inks are very complex compounds in terms of their composition, as they contain along with pigments or dyes, other ingredients generally referred to as vehicles. These materials include: humectants (to control drying), pH modifiers, polymeric resins (for proper binding), anti-foaming agents to regulate foam efficiency, wetting agents such as surfactants (which control surface properties), biocides (to inhibit microbial growth) and thickeners (to control the application and flow of the ink) [2].

Wastewater discharged from printing processes and other related operations has been reported to contain potentially hazardous components such as residual

chemicals, dyestuff, solvent residues, pigmented wiping materials, and some toxic heavy metals such as silver (Ag), copper (Cu), zinc (Zn), chromium (Cr), cadmium (Cd), lead (Pb), etc. Metals, apart from the naturally occurring ones, are products of anthropogenic activities such as chemical manufacturing, pigment and dye production, battery manufacturing, automobiles and petrochemicals. All these represent some of the major sources of input of metals into the environment [3]. Pigments used in printing operations, especially the inorganic pigments are usually metallic salts precipitated from solutions; in addition, the organic pigments also contain some metallic compounds in their chemical structures [4]. This makes it possible for bacteria in wastewater generated by printerries to develop metal resistance as a means of coping with the toxicity of metals in the wastewater. Thus wastewaters from printing operations are potential sources of metal-resistant bacteria. This feature notwithstanding has its application in the use of bacteria for metal clean up and bioremediation of metal-contaminated environments.

Although metal resistance has been widely studied in relation to industrial wastewater in Nigeria, none of

the studies has investigated printing industry wastewater as a source of metal-resistant bacteria. In addition, no study has investigated the genes responsible for metal resistance in bacteria isolated from wastewater generated by printing industries. The objective of this study was to isolate metal-resistant bacteria from wastewater collected from small and medium scale printing industries in Ibadan, Southwestern Nigeria and assess the incidence of genes encoding resistance to metals in these bacteria.

2. Materials and methods

2.1. Study site

Wastewater samples were collected from 10 printeries located in Mokola, an area which is a hub for small and medium scale printing operations and the University of Ibadan printery, both located in Ibadan, Oyo State, Nigeria. The high concentration of printeries at Mokola was responsible for the selection of the area for sample collection. The University of Ibadan printery is located within the University of Ibadan premises. The printeries collect their wastewater in holding tanks which are emptied into a central drainage channel which connects the entire community to the Ogunpa River in Ibadan, Oyo state, Nigeria.

2.2. Wastewater sample collection

Wastewater samples were collected into pre-sterilized sample containers from the final effluent holding tanks of the printeries and transported in ice chests to the Environmental Microbiology and Biotechnology Laboratory, Department of Microbiology, University of Ibadan. Samples were analysed within six hours of collection.

2.3. Metal composition of printing press industry wastewater

The metal composition of selected printing wastewater was determined using the Atomic Absorption Spectrophotometer (AAS) (UNICAM 929, London Atomic Absorption Spectrophotometer powered by SOLAAR software). The wastewater samples were digested using the nitric acid method [5]. The digested filtrates were then analysed using the cathode lamp of each metal.

2.4. Isolation of bacteria from printing wastewater

Aliquots of serially diluted printing wastewater were plated on nutrient agar (Pronadisa Laboratorios Conda, SA) using the standard pour plate technique. Morphologically distinct colonies of bacteria growing on the plates were repeatedly streaked on fresh plates to obtain pure cultures which were stored in 15% glycerol stock at -80°C for further studies.

2.5. Minimum Inhibitory concentration (MIC) of the metals on bacteria

The isolated bacteria were subjected to increasing concentrations of selected metals on Mueller Hinton agar supplemented with filter-sterilized soluble salts of CuSO_4 , PbNO_3 , CdCl_2 , $\text{K}_2\text{Cr}_2\text{O}_7$, AgNO_3 and ZnSO_4 . The starting concentration for each metal was $50\ \mu\text{g/mL}$. The culture growing on the last concentration was transferred to the next higher concentration until the isolates failed to show visible growth. The Minimum Inhibitory Concentration (MIC) was taken as the lowest concentration of the metals that prevented the growth of the bacteria [6,7].

2.6. Identification of the metal-resistant bacteria

The isolates were identified using PCR amplification/sequencing of the 16S rRNA [8]. The PCR products were sequenced (Inqaba Biotech, South Africa) and the sequences were blasted against reference sequences in the GenBank for identification (<http://www.ncbi.nlm.nih.gov/BLAST/>). Extraction of the DNA was carried out using the ZR 96 Fungal/Bacterial DNA Kit (Zymo Research Corporation, USA). The sequences were submitted to the GenBank and accession numbers were assigned.

2.7. PCR amplification of metal-resistance genes/detection of plasmids

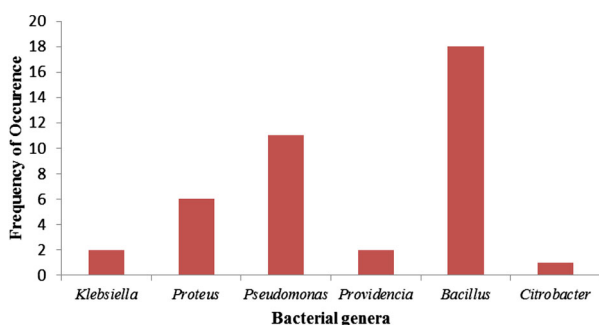
Metal-resistance encoding genes were amplified by PCR with primers targeting the chromium-zinc-cadmium resistance genes *czcA*, *czcB*, and *czcD*; silver resistance genes *silCBA*, *agrCBA* and *cusCBA*; copper resistance genes *pcoA* and *pcoR*; chromate resistance genes *chrA* and *chrB*, and lead resistance gene *pbrA*. The reaction mixture in each case contained $12.5\ \mu\text{L}$ of Master Mix, $7.5\ \mu\text{L}$ of PCR quality (Nuclease-free) water, $1.0\ \mu\text{L}$ each of both forward and reverse primers and $3\ \mu\text{L}$ of the DNA template. The annealing temperature for the PCR assays are as follows: 57°C (*czcA*, *czcB*, *czcD*, *pcoA*, *pcoR*, *chrA* and *chrB*), 55°C (*silCBA*, *agrCBA* and *cusCBA*) and 58°C (*pbrA*). All reactions included a negative (sterile water) control and a positive control where available. The oligonucleotide primers used in this study are shown in Table 1. Plasmid DNA was extracted from the bacteria using the alkaline-lysis method [9]. This was necessary because most of the genes targeted (unless stated otherwise) were plasmid-based. Plasmid sizes were determined by comparison with a DNA marker (Thermo Scientific).

3. Results

A total of 40 bacteria showing different levels of resistance to five selected metals were obtained. The

Table 1. Oligonucleotide primers used in this study.

Target genes	Primer (Forward and Reverse)	Length of amplified region (bp)	Reference
<i>pbrA</i>	5' ATGAGCGAATGTGGCTCGAAG 3' 5' TCATCGACGCAACAGCCTCAA 3'	Approx2400	[10]
<i>czcA</i>	5' GTTTGAACGTATCATTAGTTTC 3' 5' GTAGCCATCCGAAATATTCG 3'	1885	[11]
<i>czcB</i>	5' CTATTTGAAACAACAAAAGG 3' 5' CTTCAGAACAAAAGTGG 3'	1520	[11]
<i>czcD</i>	5' CAGGCTACTGACACGACCAT 3' 5' CATGCTGATGAGATTGATGATC 3'	398	[11]
<i>chrA</i>	5' CTTATACGCTACGCCAAGCTG 3' 5' GTAATGGCATTTCAGTCGCTTG 3'	1292	[11]
<i>chrB</i>	5' GTCGTTAGCTTGCCAAATC 3' 5' CGGAAAGCAAGATGTCGATCG 3'	450	[11]
<i>silCBA</i>	5' GGATCTTGCCACGAATCATATAGC 3' 5' ACGTCAGCGTGGAGTTGATGA 3'	3562	[12]
<i>agrCBA</i>	5' GCCGAAACACGACGTTCTACT 3' 5' CCCAGGAGTGAGCTTCTCATT 3'	3277	[12]
<i>cusCBA</i>	5' GATCTTAGACGGAGGCTTTATGTCATTCC 3' 5' GATCCTGCAGCAAACCATCCCGTCTGC 3'	6413	[12]
<i>pcoR</i>	5' CAGGTCGTTACCTGCAGCAG 3' 5' CTCTGATCTCCAGGACATATC 3'	636	[13,14]
<i>pcoA</i>	5' CGTCTCGACGAACTTCTCTG 3' 5' GGACTTCACGAAACATTCCC 3'	1791	[13,14]

**Figure 1.** Frequency of occurrence of metal-resistant bacteria from selected printerries.

16S rRNA gene sequencing showed that the isolates belonged to six genera namely: *Bacillus* (18), *Pseudomonas* (11), *Proteus* (6), *Klebsiella* (2), *Providencia* (2) and *Citrobacter* (1) as shown in Figure 1.

Table 2 shows the mean metal concentration of wastewater obtained from 3 selected printerries of the 10 sampled. The copper concentration of the wastewater was highest for PPW1 and PPW2 (3.07 and 4.52 mg/L respectively), while zinc was highest in PPW3 (2.22 mg/L) in comparison to the other metals. The least concentration of metal was silver for all the wastewater sampled. In most of the cases, the metal concentrations

Table 2. Mean metal concentration of wastewater of three selected printerries (mg/L).

Metal	PPW1	PPW2	PPW3	NERS Limit
Cu	3.07 ± 0.03	4.52 ± 0.03	1.42 ± 0.00	0.5
Pb	4.12 ± 0.08	0.53 ± 0.01	0.15 ± 0.05	0.1
Zn	1.12 ± 0.03	4.08 ± 0.14	2.22 ± 0.01	2
Cr	1.31 ± 0.00	0.83 ± 0.02	0.13 ± 0.00	0.5
Cd	2.22 ± 0.03	1.44 ± 0.00	0.13 ± 0.03	0.02
Ag	0.0042 ± 0.00	0.0062 ± 0.00	0.0016 ± 0.00	-

Note: PPW: Printery wastewater, NERS: National Environmental Regulation Standard in Nigeria.

were more than the National Environmental Regulations (NER) limit [15], except in few instances.

3.1. Minimum inhibitory concentration (MIC) of the metals on the bacteria

The bacteria showed varying degree of tolerance to the tested metals. *Bacillus stratosphericus* PW1b showed the highest level of tolerance to copper (Cu) with an MIC of (650 µg/mL), while the MIC for all the other bacteria ranged from 100 to 500 µg/mL. In the case of lead (Pb), 5 of the isolates were resistant at concentrations < 500 µg/mL with the remaining having MIC values ranging from 500 to 550 µg/mL. At a concentration of 500 µg/mL, 37.5% (15) of the total bacteria obtained in this study were able to grow on zinc. However, the MIC range for zinc was between 100 and 400 µg/mL for the remaining isolates. Fourteen of the 40 metal-resistant bacteria, representing 35% were resistant to cadmium with MIC value of 500 µg/mL. With the exception of *Proteus mirabilis* PW4c (MIC: 0 µg/mL) and *Pseudomonas aeruginosa* PW5c (MIC: 450 µg/mL) all the other bacteria showed a MIC ranging between 100 and 400 µg/mL for cadmium. Twenty-six of the total bacteria (65%) grew in the presence of 400 µg/mL of silver, with the rest growing at MIC < 400 µg/mL (Table 3).

3.2. Detection of metal resistance genes and plasmid profile

The silver resistance genes, *silCBA* and *agrCBA*, and the chromium-zinc-copper resistance genes *czcA*, *czcB* and *czcD* were not detected in any of the isolates. However, copper resistance genes, *pcoA* and *pcoR* were both detected in *Bacillus stratosphericus* PW1b. *chrA* and *chrB* encoding chromium resistance were detected in three bacteria (*Proteus mirabilis* PW3a,

Table 3. Minimum Inhibitory Concentration (MIC) of the metals on the bacteria ($\mu\text{g/mL}$), metal-resistance genes detected and presence of plasmids.

Bacterial isolate	Source	Pb	Zn	Cd	Cu	Cr	Ag	Metal-resistance genes detected	Plasmid	Accession Number
<i>Bacillus aerius</i> PW1a	PP1	500	500	400	500	200	400	<i>cusCBA</i>	Yes	MK026845
<i>Klebsiella oxytoca</i> PW1ay	PP1	500	500	400	500	400	400	NMGD	Yes	MK026847
<i>Bacillus stratosphericus</i> PW1b	PP1	250	100	100	650	100	100	<i>cusCBA, pcoA, pcoR</i>	Yes	MK026844
<i>Pseudomonas japonica</i> PW1c	PP1	500	100	300	400	100	400	NMGD	Yes	MK026846
<i>Bacillus stratosphericus</i> PW1e	PP1	500	200	500	500	500	400	NMGD	No	MK026849
<i>Bacillus aerophilus</i> PW2a	UIP	100	100	100	100	100	100	<i>cusCBA</i>	Yes	MK026850
<i>Providencia vermicola</i> PW2b	UIP	500	100	100	100	200	300	<i>cusCBA</i>	Yes	MK026875
<i>Bacillus stratosphericus</i> PW2bb	UIP	450	300	350	300	400	200	<i>cusCBA</i>	Yes	MK026843
<i>Proteus mirabilis</i> PW3a	PP2	500	500	300	200	400	300	<i>chrA</i>	Yes	MK123476
<i>Bacillus aerophilus</i> PW3c	PP2	500	100	100	100	100	400	<i>cusCBA</i>	Yes	MK026864
<i>Pseudomonas aeruginosa</i> PW3d	PP2	500	200	100	500	400	400	<i>pbrA, chrA</i>	Yes	MK123475
<i>Klebsiella oxytoca</i> PW4a	PP3	500	100	100	400	500	400	<i>cusCBA</i>	Yes	MK026848
<i>Proteus mirabilis</i> PW4b	PP3	500	100	200	100	400	400	NMGD	Yes	MK026861
<i>Proteus mirabilis</i> PW4c	PP3	500	100	NG	100	400	100	<i>cusCBA, chrB</i>	Yes	MK026860
<i>Pseudomonas aeruginosa</i> PW4d	PP3	500	400	500	400	100	100	<i>pbrA</i>	Yes	MK026858
<i>Pseudomonas aeruginosa</i> PW5a	PP4	500	400	500	200	100	NG	NMGD	Yes	MK026869
<i>Bacillus cereus</i> PW5b	PP4	550	400	400	500	400	400	NMGD	Yes	MK026856
<i>Pseudomonas aeruginosa</i> PW5c	PP4	350	300	450	350	450	200	<i>pbrA, chrA</i>	Yes	MK026867
<i>Pseudomonas aeruginosa</i> PW5d	PP4	500	400	500	400	100	100	<i>pbrA</i>	Yes	MK026853
<i>Proteus mirabilis</i> PW5e	PP4	500	400	400	500	NG	400	<i>cusCBA</i>	Yes	MK026870
<i>Bacillus aerophilus</i> PW5f	PP4	500	500	100	500	500	300	NMGD	Yes	MK026842
<i>Bacillus thuringiensis</i> PWA1	PP5	500	200	400	100	400	400	NMGD	Yes	MK026865
<i>Proteus mirabilis</i> PWA2	PP5	500	200	500	500	200	400	NMGD	Yes	MK026868
<i>Bacillus cereus</i> PWA3	PP5	500	500	300	500	400	400	NMGD	Yes	MK026877
<i>Citrobacter freundii</i> PWA4	PP5	100	100	100	100	100	-	NMGD	No	MK026871
<i>Pseudomonas aeruginosa</i> PWAP1	PP6	500	500	500	500	100	300	<i>pbrA</i>	Yes	MK026872
<i>Bacillus cereus</i> PWAP2	PP6	500	500	500	500	200	400	NMGD	Yes	MK123474
<i>Providencia vermicola</i> PWAP3	PP6	500	500	500	400	200	400	<i>chrB</i>	Yes	MK026862
<i>Bacillus aerophilus</i> PWN1A	PP7	500	500	400	500	200	400	NMGD	No	MK026857
<i>Bacillus thuringiensis</i> PWN1B	PP7	500	200	300	500	400	400	NMGD	No	MK026866
<i>Bacillus subtilis</i> PWN1C	PP7	500	500	500	500	200	400	NMGD	Yes	MK026874
<i>Bacillus thuringiensis</i> PWN2A	PP8	500	200	500	500	200	400	NMGD	Yes	MK026851
<i>Bacillus thuringiensis</i> PWN2B	PP8	500	500	500	500	500	400	NMGD	Yes	MK026873
<i>Pseudomonas aeruginosa</i> PWN2C	PP8	500	400	500	400	100	300	<i>pbrA</i>	Yes	MK026852
<i>Bacillus thuringiensis</i> PWN2D	PP8	500	500	300	500	500	400	NMGD	Yes	MK026854
<i>Proteus mirabilis</i> PWN3A	PP9	500	200	400	100	400	400	NMGD	Yes	MK026855
<i>Bacillus cereus</i> PWN3B	PP9	500	500	500	500	500	400	NMGD	Yes	MK026863
<i>Pseudomonas aeruginosa</i> PWN3D	PP9	500	500	500	500	200	400	<i>pbrA</i>	Yes	MK026876
<i>Pseudomonas aeruginosa</i> PWN3T	PP9	400	500	400	500	400	400	NMGD	Yes	MK026859
<i>Pseudomonas aeruginosa</i> PWN3Y	PP9	500	400	400	400	300	400	NMGD	Yes	MK026878

KEY: PP: Printery, UIP: University of Ibadan Printery, NG: No growth was observed after the incubation period, NMGD: No metal-resistance gene detected, Yes: Presence of plasmid, No: Absence of plasmid.

Pseudomonas aeruginosa PW5c and *Pseudomonas aeruginosa* PW3d) and two bacteria (*Providencia vermicola* PWAP₃ and *Proteus mirabilis* PW4c) respectively. *cusCBA* was detected in nine isolates namely: *Proteus mirabilis* PW4c, *Bacillus aerius* PW1a, *B. stratosphericus* PW2bb, *Bacillus aerophilus* PW2a, *Klebsiella oxytoca* PW4a, *Proteus mirabilis* PW5e, *Providencia vermicola* PW2b, *Bacillus aerophilus* PW3c and *Bacillus stratosphericus* PW1b. *pbrA* was detected in seven *Pseudomonas aeruginosa* strains spread across the sampling sources. The plasmid profile showed that 36 of the 40 metal-resistant bacteria possessed plasmids with sizes larger than 10Kb (Figure 2).

4. Discussion

Various genera of metal-resistant bacteria have been isolated from different sources notably wastewater. Several authors have reported the isolation of metal-resistant bacteria from various sources [7,16–18]. Bacteria adapt to metal stress in their environment and respond to it by developing several resistances or

coping mechanisms to its toxicity [19]. This has made the study of bacteria in metal-contaminated environment an interesting one. Furthermore, the presence of metal contaminants in the immediate environment could also act as a precursor in the stimulation of resistance to metal species by bacteria. The printing press wastewater employed in this study had a considerable level of metal contaminants present, and this could have propelled the bacteria therein to develop adaptive features against those metals.

The *cop* resistance determinants which share a functional similarity with *pco* resistance determinants have been confirmed to be responsible for copper resistance in *Pseudomonas syringae* [20], whereas *pco* are responsible for copper resistance in *Escherichia coli*. In contrast however, other researchers reported that there is a slight difference in the mechanisms of action of *cop*- and *pco*- encoded copper resistance in bacteria. The *cop* genes are believed to encode the sequestration of copper and higher accumulation [21], whereas that encoded by *pco* is an energy-dependent export and lower accumulation of copper in the bacterial

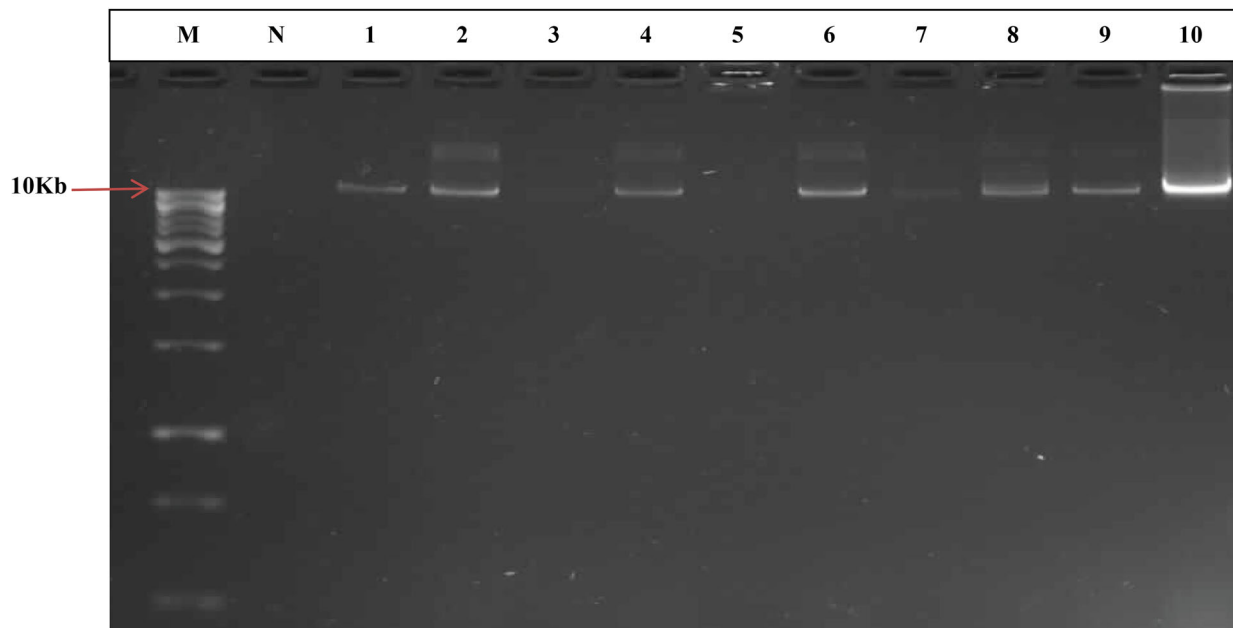


Figure 2. Plasmid profile of selected metal-resistant bacteria on 0.8% agarose gel. Lanes represent the following: M: DNA marker (Thermo Scientific), N: Negative control, 1: *B. cereus* PW5b, 2: *P. mirabilis* PWN3A, 3: *B. aerophilus* PWN1A, 4: *B. thuringiensis* PWN2B, 5: *B. stratosphericus* PW1e, 6: *B. cereus* PWA3, 7: *B. stratosphericus* PW2bb, 8: *B. subtilis* PWN1C, 9: *B. thuringiensis* PWN1B, 10: *P. aeruginosa* PWAP1.

cell [22,23]. The proposed mechanism of copper resistance in *Escherichia coli* requires the cooperation of both the plasmid and chromosomal functions to initiate resistance in an integrated fashion [24]. Plasmid-mediated resistance to copper has been reported in several species of bacteria especially *Pseudomonas syringae* pv tomato and *E. coli* and documented by several authors [14,24–28]. On the contrary however, chromosomal resistance to copper has also been described in *Enterococcus hirae* [29,30]. This might be responsible for the phenotypic resistance to copper by some bacteria in this study, even without the possession of the copper resistance determinants.

The range of bacterial hosts in which the *pco* determinant could function, might be limited to those genera closely related to *E. coli*, such as *Citrobacter*, *Salmonella*, and *Shigella*, all Gram negative organisms. This is in sharp contrast with the findings from this study, in which *Bacillus stratosphericus* PW1b, a totally unrelated Gram positive organism, was found to possess the *pco* gene determinants. The presence of these genes in gram positive group of bacteria could be attributed to the plasmid-borne nature of the gene which might have broadened its host spectrum [24]. Though Chihomvu and his co-workers in 2015 in their study on Klip River in South Africa, reported the detection of the copper resistance gene, *pcoA* in *Lysinibacillus* sp. KR25, this study is the first report of the detection of *pco* genes in any Gram positive bacterium isolated from printing wastewater.

The *chrBAC* operon is a set of genes harboured by the pMOL28 plasmid of the multi-metal resistant *Cupriavidus metallidurans* CH3. *chrA* chromate resistance protein has been detected in strains of *Pseudomonas*

aeruginosa [31]. Two *Pseudomonas* strains in this study were observed to possess the *chrA* gene; however *Proteus mirabilis* PW3a in this study was also detected to possess the same gene. Based on the literatures at our disposal, this is likely to be the first report of the detection of *chrA* gene in *Proteus mirabilis* especially from printing wastewater. In addition, it has been reported that bacterial resistance to chromium may be due to either chromosomal mutations [32] or plasmid-mediated [33,34]. The presence of plasmids encoding chromate resistance has also been reported in certain species of *Pseudomonas*, *Alcaligenes*, *Salmonella*, *Bacillus* and *Escherichia coli* by several authors [34–39]. In this present study, *chrB* which regulates the *chrA* transporter [40] was detected in *Providencia vermicola* PWAP3 and *Proteus mirabilis* PW4c and this corroborated the report on the possession of the *chr* operon on the plasmids of species of Gram negative bacteria [41]. The same authors also reported the detection of the genes in some *Bacillus* strains isolated from tannery effluent.

The CBA-transport systems which are involved in the export of metal ions, xenobiotics and drugs are exclusively found in Gram negative bacteria. The need for Gram negative cells to safeguard the cytoplasm and translocate metals and other toxicants across their outer membrane has necessitated this system. Contrary to this report however, the *cusCBA* was detected in some strains of bacteria that are not Gram negative in this study. Five of the seven bacteria possessing the *cusCBA* in this study were gram-positive, while the remaining belongs to the gram-negative genera. The 6413 bp gene was detected in *Proteus mirabilis* PW4c, *Bacillus aerius* PW1a, *Bacillus stratosphericus* PW2bb,

Bacillus aerophilus PW2a, *Klebsiella oxytoca* PW4a, *Proteus mirabilis* PW5e, *Providencia vermicola* PW2b, *Bacillus aerophilus* PW3c, and *Bacillus stratosphericus* PW1b. The gene which has also been detected in *Escherichia coli* is also carried by the pMOL30 plasmid of the well-studied, multi-metal resistant *Cupriavidus metallidurans* CH34 [42,43].

All the *Pseudomonas* strains possessing the *cusCBA* in this present study showed varying resistance to copper and silver as outlined by their MIC to copper and silver ions; and this corroborates the report of some authors who opined that the *cus* determinant is induced by copper and silver, though the inducement by silver is to a lesser extent compared to copper [44–46]; Their findings were partly corroborated by other reports on the clear contribution of the *cus* to copper resistance under anaerobic condition [46], they went further to report that the detoxification of copper by the *cus* system occurred in an oxygen-rich atmosphere. Contrary to this however, it has been reported that the protein of the *cus* system only mediates resistance to silver and that the conferment of resistance to copper could not be ascertained, even when the *copA* which encodes the copper-detoxifying P-type ATPase was disrupted in a mutant background. They however showed in their finding that copper was a better inducer of the expression of *cus* than silver, suggesting a probable involvement of the genes in the resistance of bacteria to copper; as reported by other researchers [47,48].

The *pbr* proteins are a group of proteins encoded in the widely studied metal-resistant *Cupriavidus metallidurans* CH34, and they include; *PbrT*, *PbrA*, *PbrB*, *PbrC*, *PbrD* and *PbrR*. The *pbrA*, is a PIB-type ATPase in *Cupriavidus metallidurans*, and is the main lead efflux transporter [10]. The gene was detected in 7 of the 40 (17.5%) metal-resistant bacteria obtained in this study. The bacteria found to possess the *pbrA* gene were all strains of *Pseudomonas aeruginosa*. Strains of *Pseudomonas marginalis* and *Bacillus megaterium* have been observed to show extracellular lead exclusion and intracellular cytoplasmic lead accumulation respectively. Pb-resistant strains of other bacteria e.g. *Staphylococcus aureus*, *Citrobacter freundii* and *Vibrio harveyi* have also been reported [49–52].

Though it was initially thought that the Pbr efflux system was Pb(II) specific. The participation of the Pbr efflux in the protection of the cell wall against Cd (II) and Zn (II) has been reported [53]. The specific mechanisms of Pb(II) resistance require the mutual cooperation of *pbrA* and *pbrB* genes hence the two are majorly involved in mediating lead resistance in bacteria e.g. in the metal-resistant *Cupriavidus metallidurans* [10]. It should be stressed however that the functional roles of the other genes in the Pbr efflux system e.g. *PbrT*, *PbrC* and *PbrD* are still an issue of debate among researchers, because their absence does not in any way impair the ability of the system to neutralize toxic ions

in *Cupriavidusmetallidurans* [10,53] However from this present study, all the bacteria were able to tolerate different concentration of lead, even without the possession of the *pbrA*. This suggests that there might probably be other mechanisms of resistance to the metal possessed by the strains and the possibility of the resistance being chromosome-mediated or being mediated by other gene variants.

Conclusion

This study has highlighted printing industries in Ibadan, Nigeria as a potential contributor of metal-resistant bacteria and their genes into the environment, highlighting an urgent need for the enforcement of regulations regarding wastewater discharge, especially in developing countries of the world where wastewater from the manufacturing sector is discharged into the environment without prior treatment.

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