NATIVE CHROMIUM RESISTANT *Staphylococci* SPECIES FROM A FLY ASH DUMPING SITE IN SOUTH AFRICA HARBOR PLASMID pMOL28 DETERMINANTS

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ABSTRACT

Sixty-six chromium-resistant *Staphylococci* species belonging to *S. epidermidis*, *S. aureus, S. saprophyticus* and *S. arlettae* were previously isolated from a chromium-polluted Fly ash (FA) dumping site in South Africa. However the genetic mechanisms responsible for chromium resistance were not known. Polymerase chain reaction and DNA-DNA hybridization techniques were used to explore whether or not these staphylococcal strains exhibited *chr* determinants of plasmid pMOL28 that confers chromate resistance to *Cupriavidus metallidurans*. Genes $ChrB_1$ and *ChrC* were found to be present in the genomes of all the *Staphylococci* species. However, gene $ChrA_1$ was shown to be present only in the genome of *S. aureus*. These results showed that *Chr* genes could be distributed from *Cupriavidus metallidurans* (Gram negative bacteria) to *Staphylococci* species (Gram positive bacteria), probably by horizontal transfer.

Keywords : Fly Ash, chromium resistant bacteria, *Staphylococci*, *Chr* determinants, pMOL28.

RESUME

DIFFERENTES ESPECES DE Staphylococci *POSSEDANT LES GENES DU PLASMIDE PMOL28*

Soixante-six Staphylococci *résistants au chrome appartenant aux espèces :* S. epidermidis, S. aureus, S. saprophyticus *et* S. arlettae *ont été préalablement isolés d'un site d'enfouissement de cendres volantes pollué au chrome en Afrique du Sud. Les mécanismes génétiques responsables de la résistance au chrome chez ces bactéries n'étant pas connus, des méthodes utilisant la PCR et des techniques d'hybridation ADN-ADN ont été utilisées pour voir si oui ou non ces souches de staphylocoques arboraient les déterminants* chr *du plasmide pMOL28 qui confère la résistance au chrome chez* Cupriavidus metallidurans*. Par utilisation de ces techniques, il a été montré que les gènes* $ChrB_1$ *et* ChrC *sont présents dans les génomes de toutes les espèces de* Staphylococci*. Cependant, la présence du gène* $ChrA_1$ *a été mise en évidence que dans le génome de* S. aureus*. Ces résultats montrent que les gènes* Chr *pourraient être distribués à partir de* Cupriavidus metallidurans *(bactérie à Gram négatif) jusqu'aux espèces de staphylocoques (bactéries à Gram positif), probablement par transfert horizontal.*

Mots clés : *Cendres volantes, bactéries résistantes au chrome,* Staphylococci*, déterminant* Chr*, pMOL28.*

INTRODUCTION

The dissemination of heavy metals through the environment can be either a natural phenomenon or caused by human activities (Mc Donald and Grandt 1981; Alloway, 1995). Of all environmental pollutions, the ones due to chromium are the most widespread because of a larger number of industrial applications (Barceloux, 1999). This has largely contributed to the occurrence of high chromium (Cr) concentrations in polluted areas (Nriagu, 1988). In general, bacteria can survive chromium toxicity through 4 mechanisms : (a) the transmembrane efflux of chromate (Cervantes *et al.,* 1990 ; Nies *et al.,* 1990) (b) the ChrR transport system (Saier, 2003), (c) the reduction of chromate (Cervantes and Campos, 2007), (d) the protection against oxidative stress (Ackerley *et al.,* 2006 ; Brown *et al.*, 2006 ; Henne *et al.,* 2009) and the DNA repair systems (Miranda *et al.*, 2005, Chourey *et al.*, 2006). The most studied efflux pump in chromate resistance is conferred to bacteria by the ChrA transporter encoded by plasmid pMOL28 in *Cupriavidus metallidurans* (Nies *et al.,* 1990). Plasmid pMOL28 carries genes *chrI, chrB*$_1$*, chrA*$_1$*, chrC, chrE* and *chrF1*. It has been demonstrated that, the chromate resistance determinants located on plasmid pMOL28 evolved by gene duplication and horizontal gene transfer event (Von Rozycki and Nies, 2008). Gene transfer process is the main basis of heavy metal resistance (HMR) acquisition within bacterial communities (Mergeay, 2000 ; Nies, 1999 ; Silver and Phung, 1996). That has allowed the dissemination of surviving phenotypes into bacterial communities (Osborn *et al.*, 1997 ; Coombs and Barkay, 2004). In a previous work, we identified four chromium resistant *Staphylococci* species isolated from a Fly Ash dumping site in South Africa (*S. Aureus, S. epidermidis, S. arlettae, S. saprophyticus*), having the potential to resist this metal (Kouadjo and Zeze, 2011). However, the mechanisms through which these Grams positive bacteria survive within this highly polluted chromium environment were not elucidated. In *Cupriavidus metallidurans* (Gram negative bacteria), it was shown that the transcription of genes *ChrA*$_1$*, ChrB*$_1$ and *ChrC* was induced by chromate (Juhnke *et al.,* 2002). Transfer of chromium resistance determinants has been already observed between Gram negative and Gram positive bacteria (Abou-Shanab *et al.,* 2007). Do the Gram positive chromium resistant *Staphylococci* species from the fly ash dumping site harbor the plasmid pMOL28, mainly genes *ChrA*$_1$*, ChrB*$_1$ and *ChrC?* It was important to characterize the system used by these bacteria to resist chromium toxicity. The objective of this study is to show, whether or not the chromium resistant *Staphylococci* isolated from this FA dumping site harbored the *Cupriavidus metallidurans* genes *ChrA*$_1$, *ChrB*$_1$ and *ChrC* located on the megaplasmid pMOL28.

MATERIAL AND METHODS

BACTERIAL STRAINS

The Chromium resistant bacteria (CRB) used in this study were isolated in a previous work, from different depths within a FA dumping site, containing various chromium concentrations (Kouadjo and Zézé, 2011). 16S rRNA and phylogeny analyses, allowed the identification of 5 species of which 4 were selected for this study (Table 1).

Table 1 : Staphylococci species used in this study.

Différentes espèces de Staphylococci utilisées dans cette étude.

Strain	Identification	Number of Strain	Depth occurrence within the FA dumping site
10m-55	*S. saprophyticus*	26	10m
3m-3	*S. arlettae*	34	3m
3m-6	*S. epidermidis*	04	3m
7m-11	*S. aureus*	02	7m

GENOMIC DNA EXTRACTION

Total genomic DNA was extracted directly from bacterial cultures according to method described by Simmons and Norris (2002). Bacterial cells were collected from 250 ml culture by centrifugation, and mixed with proteinase K (100 mg ml^{-1}) and 20 % SDS (Sodium Dodecyl sulfate). DNA extraction with phenol-chloroform-isoamyl was followed by precipitation and washing. The purified genomic DNA was used as template in PCR and hybridization reactions.

PCR AMPLIFICATION OF CHROMIUM RESISTANT GENES *ChrA*$_1$, *ChrB*$_1$ AND *ChrC*

In order to amplify *ChrB*$_1$ and *ChrA*$_1$, degenerated and specified primers listed in Table 2 were used, to amplify corresponding regions in the genomic DNA of isolated staphylococcal strains, including *Cupriavidus metallidurans* (CH34). PCR reaction conditions included for gene *ChrA*$_1$ amplification, a 5 min hot start step at 94 °C, followed by 30 cycles of 94 °C for 30 seconds, 59 ºC for 30 seconds and 72 ºC for 40 seconds and a final extension of 72 ºC for 10 min. The program to amplify *ChrB*$_1$ was : 94 ºC 5 min for initial denaturation, followed by 30 cycles of 94 ºC for 30 seconds, 52 ºC for 30 seconds and 72 ºC for 40 seconds and a final extension of 72 ºC for 10 min. Finally, the program to amplify gene *ChrC* from the model species using specific primers was : 94 ºC 5 min for initial denaturation, followed by 30 cycles of 94 ºC for 30 seconds, 60 ºC for 30 seconds and 72 ºC for 40 seconds and a final extension of 72 ºC for 10 min.

OBTAINING PROBES FROM CH34

Genes *ChrA*$_1$, *ChrB*$_1$ and *ChrC* obtained by PCR amplification from *C. metallidurans* (CH34), the model species, using degenerated and specified primers listed in table 2 were digoxigenin labeled by random priming according to Boehringer protocol and used as probes.

Table 2 : Primers used in this study.

Amorces utilisées dans cette étude.

Primers	Sequence 5'- 3'	Hybridization temperature
$ChrA_{1Forward}$	AAA GGT ACC TCG GTA CAT ACC GCG CCC ACT	59 °C
$ChrA_{1reverse}$	AAA TCT AGA TCA GTG ATG CAA CAA CGG ATA	
$ChrB_{1deg\ Forward}$	TGC GBG AYG GYG YYT AYC T	52 °C
$ChrB_{1deg\ reverse}$	SGC VCC RTC RAA RTC RAA	
$ChrC_{Forward}$	GGG CAA GGC GCT CGG CGG CGG	60 °C
$ChrC_{Reverse}$	TGC GCC AGG CAG CCC CCG CAA	

GENOMIC DNA HYBRIDIZATION

Genomic DNA hybridizations were performed in order to investigate the presence of the chromium resistance genes *ChrB*$_1$, *ChrC* and *ChrA*$_1$, in the genomes of isolated bacteria. Genomic DNAs extracted from the 66 staphylococcal strains, were blotted onto a positively charged nylon membrane, according to the manufacturer's procedure for slot blot hybridization. For Southern hybridization, total DNAs extracted from *S. aureus*, *S. epidermidis*, *S. arlettae* and *S. saprophyticus*, were digested with restriction enzyme (*Sau3a*), in a total volume of 50 µl at 37 °C overnight. After electrophoreses on 0.8 % agarose gel, the digested DNAs were transferred onto a nylon membrane by capillarity. After overnight hybridizations at 68 ºC, filters were washed twice for 10 min at room temperature in 2X SSC (Saline Sodium Citrate) ; 0.1 % SDS and twice for 15 min in 0.1 % SSC-0.1 % SDS. The hybridization signals were detected by the chemiluminescence system (Boehringer) and exposure of the membrane to a medical X-Ray film.

PHYLOGENETIC ANALYSES

16S rRNA sequences from databases belonging to different strains of *C. metallidurans* and those obtained from the CR *Staphylococci* species (*S. aureus*, *S. epidermidis*, *S. arlettae* and *S. saprophyticus*) by Kouadjo and Zézé (2011) were used to construct a phylogenetic tree. The

phylogenetic tree was constructed, using the neighbor joining method (Saitou and Nei, 1987) and MEGA4 software (Tamura *et al.,* 2007).

RESULTS

DETECTION OF GENE *ChrA*$_1$, *ChrB*$_1$ AND *ChrC* IN THE *STAPHYLOCOCCAL* STRAINS BY SLOT HYBRIDIZATION

Using the primers (Table 2) genes *ChrA*$_1$, *ChrB*$_1$ and *ChrC* were amplified from the genome of *C. metallidurans*. After amplification, genes *ChrA*$_1$, *ChrB*$_1$ and *ChrC* were obtained at the expected sizes (1.1 kb, 970 bp and 500 bp) respectively (Figure 1). Genes *ChrA*$_1$, *ChrB*$_1$ and *ChrC* amplified from *C. metallidurans* were then used as probes for detection by slot blot hybridization in the genomes of the 66 strains representing *S. aureus, S. epidermidis, S. saprophyticus* and *S. arlettae*. Gene *ChrC* was detected at different signal intensities in most of the 66 *staphylococcal* strains (Figure 2). This gene was present within the 4 species in different proportions (Table 3A). Out of 26 strains from *S. saprophyticus*, 20 gave positive signals that correspond to 78.57 % while 23 strains out of 34 (75 %) from *S. arlettae* gave positive signals. For *S. epidermidis* out of the 4 strains used, 3 gave positive signals while for *S. aureus* all the 2 strains gave positive signals. When gene *ChrB*$_1$ was used as probe in slot blot hybridization of the 66 strains, all of them gave a positive signal (Table 3B). When gene *ChrA*$_1$ was used as a probe in the same conditions, no hybridization signal was obtained for any strain belonging to *S. epidermidis*, *S. saprophyticus* or *S. arlettae*. Only the two strains from *S. aureus* gave a positive signal (Table 3C).

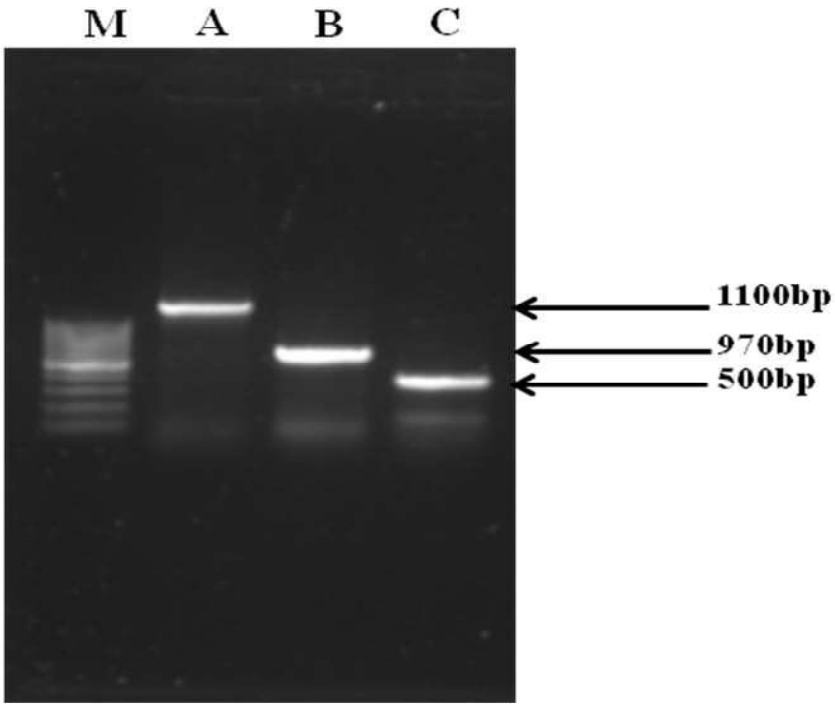

Figure 1 : Agarose gel electrophoresis (1 %) of PCR product of A) gene *ChrA1*, B) gene *ChrB1* and C) gene *ChrC* obtained from *C. metallidurans*. M) 100 bp DNA ladder.

Gel d'électrophorèse d'agarose (1 %) des produits PCR des gènes A) ChrA1*, B)* ChrB1 *et C)* ChrC *amplifiés à partir de* C. metallidurans *(CH34). M) Marqueur ADN de 100 pb.*

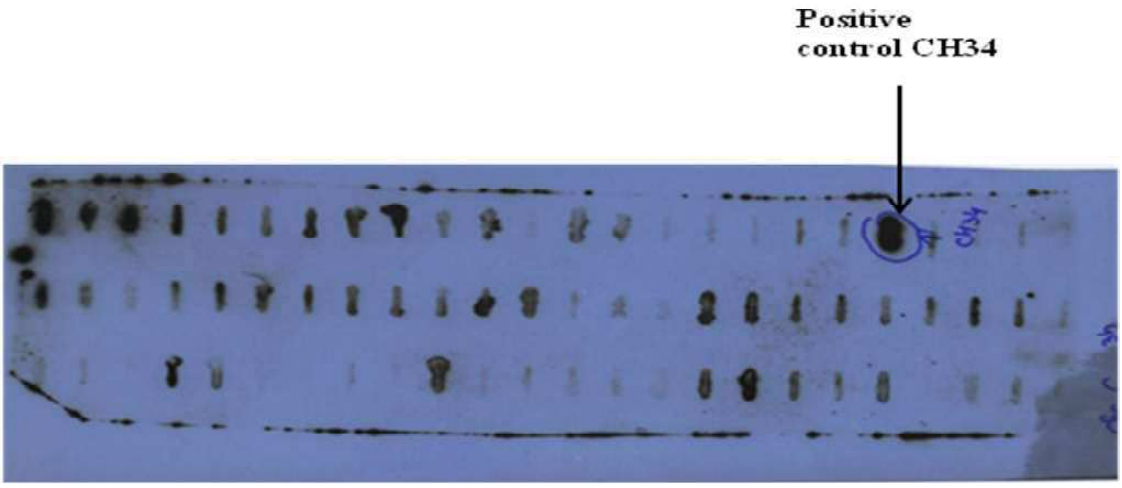

Figure 2 : Slot blot hybridization of total DNA extracted from the 66 *Staphylococcal* strains isolated from the FA dumping site, using digoxigenin labeled *ChrC* gene. Autoradiogram of hybridization show different signal intensities.

Autoradiogramme d'hybridation des ADN de 66 Staphylococci *isolés d'un site d'enfouissement de FA utilisant le gène* ChrC *comme sonde marquée à la Digoxigénine. Les signaux d'hybridation apparaissent avec différentes intensités.*

Table 3 : Distribution of A) gene *ChrC,* B) gene *ChrB1* and C) gene *ChrA1* within the 66 *staphylococci* strains according to signal intensities obtained by slot blot hybridization using these genes as probes.

Distribution des gènes A) ChrC, *B)* ChrB1 *et C)* ChrA1 *parmi les 66 souches de* Staphylococci. *La distribution est basée sur l'observation des signaux obtenus après hybridation slot blot utilisant ces gènes comme sonde.*

A) ChrC

Strains	Species	Depth	Number of strains		
			Positive signal	Negative signal	Total
10m-55	*S. saprophyticus*	10m	20	06	26
3m-3	*S. arlettae*	3m	23	11	34
3m-6	*S. epidermidis*	3m	03	01	04
7m-11	*S. aureus*	7m	02	00	02

B) $ChrB_1$

Strain	Species	Depth	Number of strains		
			Positive signal	Negative signal	Total
10m-55	*S. saprophyticus*	10m	26	00	26
3m-3	*S. arlettae*	3m	34	00	34
3m-6	*S. epidermidis*	3m	04	00	04
7m-11	*S. aureus*	7m	02	00	02

C) $ChrA_1$

Strain	Species	Depth	Number of strains		
			Positive signal	Negative signal	Total
10m-55	*S. saprophyticus*	10m	00	26	26
3m-3	*S. arlettae*	3m	00	34	34
3m-6	*S. epidermidis*	3m	00	04	04
7m-11	*S. aureus*	7m	02	00	02

DETECTION OF GENE *ChrA*$_1$ BY PCR AMPLIFICATION AND SOUTHERN HYBRIDIZATION

With presumption that gene *ChrA*$_1$ was only present in the species *S. aureus* after slot blot hybridization, primers *ChrA*$_1$forward and *ChrA*$_1$reverse (Table 2) were used to confirm this result. The genomic DNA of a representative of each *staphylococci* species (*S. epidermidis, S. aureus, S. saprophyticus* and *S. arlettae)* was used as template for PCR amplification. After PCR reaction, a PCR fragment (900 bp) different in size from *C. metallidurans* expected fragment (1 100 bp) was obtained from *S. aureus* (Figure 3). No amplification was obtained from the other species confirming the results obtained in slot blot hybridization. When genomic DNAs obtained from S. *epidermidis, S. aureus, S. saprophyticus* and *S. arlettae* were digested (Figure 4A), blotted onto a membrane and hybridized with *ChrA*$_1$ from *C. metallidurans*, again only DNA from *S. aureus* gave a signal (Figure 4B).

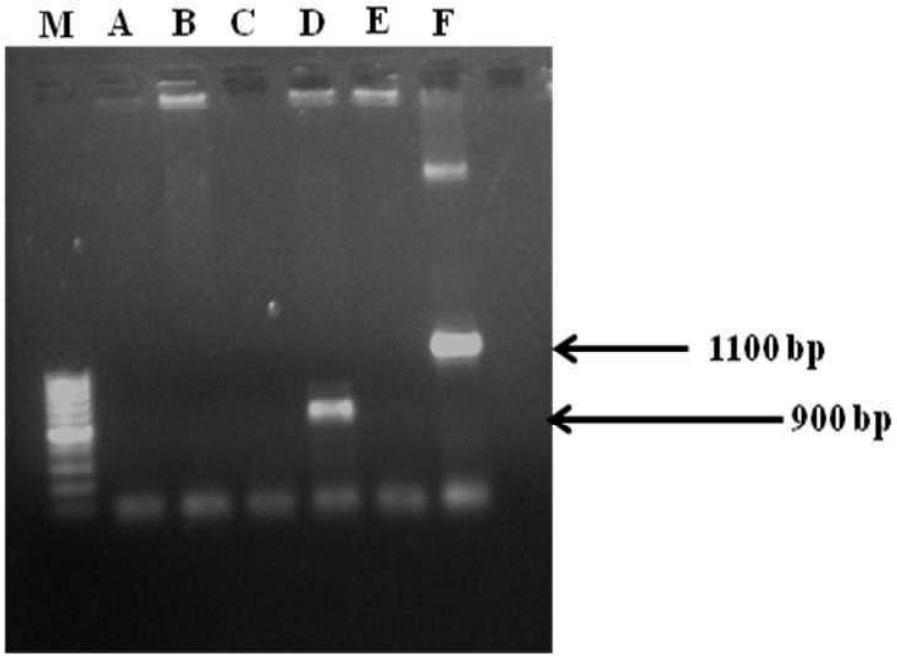

Figure 3 : Agarose gel electrophoresis (1 %) of PCR product of *ChrA1* gene obtained from A) *S. epidermidis* B) *S. arlettae,* C) *S. saprophyticus*, D) *S. aureus.* M) 100 bp DNA ladder, E) Negative control, F) Positive control.

Gel d'électrophorèse d'agarose (1 %) des produits PCR du gène ChrA1 *obtenu à partir de A)* S. epidermidis *B)* S. arlettae*, C)* S. saprophyticus*, D)* S. aureus *M) 100 pb ADN marqueur, E) Témoin négatif, F) Témoin positif.*

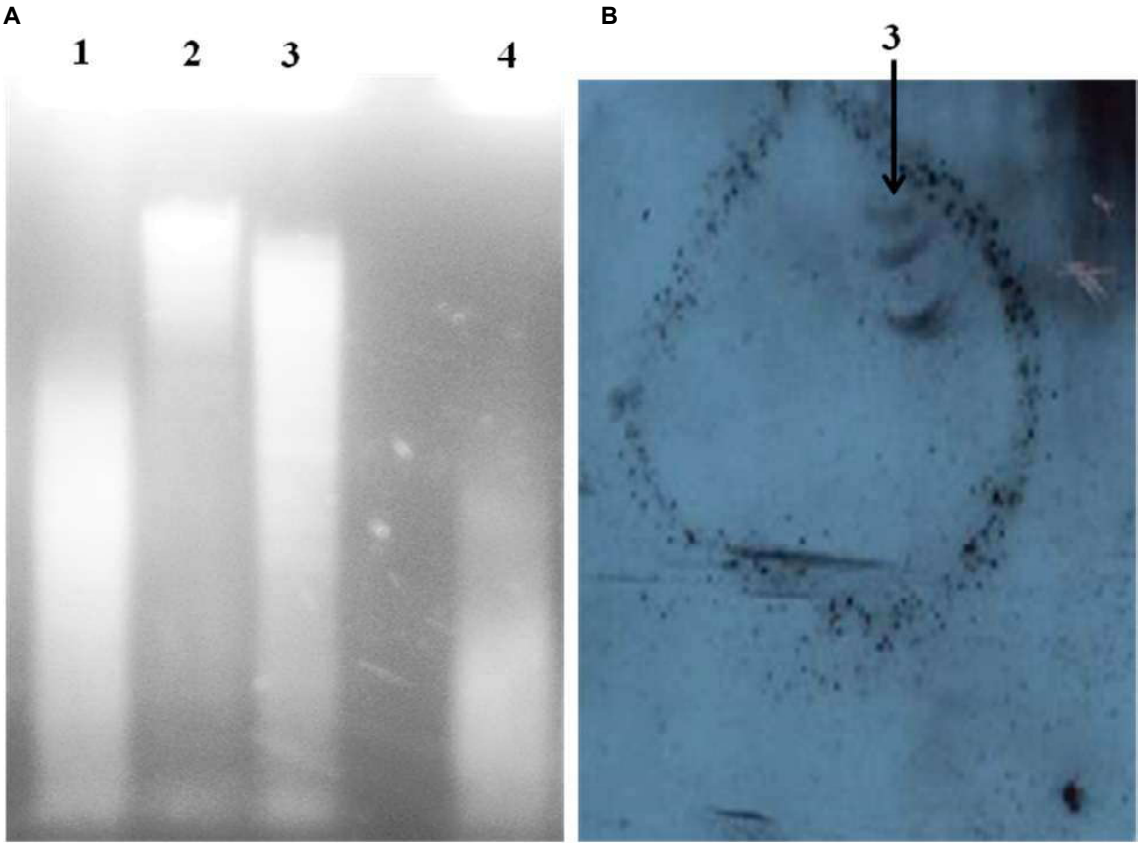

Figure 4 : (A) Agarose gel electrophoresis (1 %) of 1) *S. epidermidis* 2) *S. arlettae* 3) *S. aureus* 4) *S. saprophyticus* digested with Sau3a and B) blotted and hybridized with digoxigenin labeled *ChrA1* fragment amplified from CH34.

(A) Gel d'électrophorèse d'agarose (1 %) des ADN extraits de 1) S. epidermidis *2)* S. arlettae *3)* S. aureus *4)* S. saprophyticus *digérés avec Sau3a et B) transférés et hybridés avec le gène* ChrA1 *amplifié de CH34 et marqué à la digoxigénine.*

PCR DETECTION OF GENE *ChrB*$_1$ IN THE GENOMES OF *S. epidermidis*, *S. aureus*, *S. arlettae* AND *S. saprophyticus*

In order to confirm the presence of gene *ChrB*$_1$ revealed by slot blot hybridization, a representative of the four species (*S. epidermidis*, *S. aureus*, *S. arlettae* and *S. saprophyticus*) was used as a template to perform PCR reaction, using *ChrB*$_1$ primers listed in table 2. A PCR fragment was obtained from the genomes of the four species (Figure 5) confirming the result obtained in slot blot hybridization. Meanwhile, the PCR products did not have the same size. The PCR product obtained with *S. aureus* had the *C. metallidurans* expected PCR product size (970 bp) (Figure 5). A main PCR product of 1 100 bp was obtained with *S. saprophyticus* and *S. epidermidis* while a 1 600 bp fragment was obtained with *S. arlettae*.

Cupriavidus metallidurans AND THE *Staphylococcal* STRAINS ARE PHYLOGENETICALLY DISTANT

In order to analyze the phylogenetic relatedness, between the *C. metallidurans* CH34 and the *Staphylococci* used in this study, a phylogenetic tree based on their 16S sequences was constructed. It was shown that CH34 is a ß-*Proteobacteria*, while the *Staphylococcal* strains are in the *Bacilli* class (Figures 6 and 7).

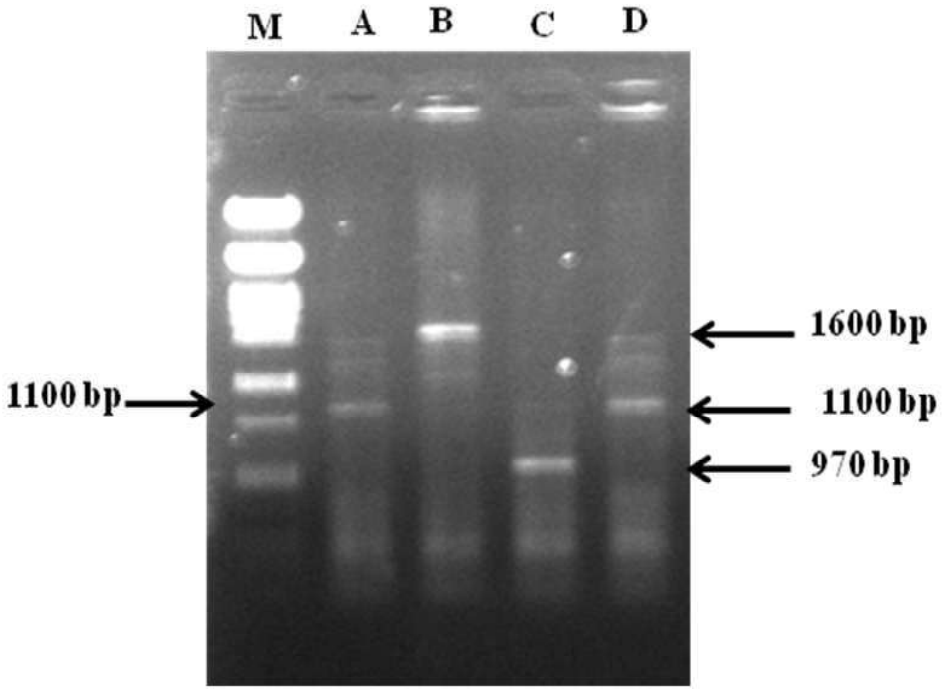

Figure 5 : Agarose gel electrophoresis (1 %) of PCR products of *ChrB1* gene obtained from A) *S. epidermidis,* B) *S. arlettae,* C) *S. aureus,* D) *S. saprophyticus* and M) 1 kb DNA ladder.

Gel d'électrophorèse d'agarose (1 %) des produits PCR du gène ChrB1 *obtenus à partir de A)* S. epidermidis, *B)* S. arlettae, *C)* S. aureus, *D)* S. saprophyticus *et M) Marqueur 1 kbp ADN.*

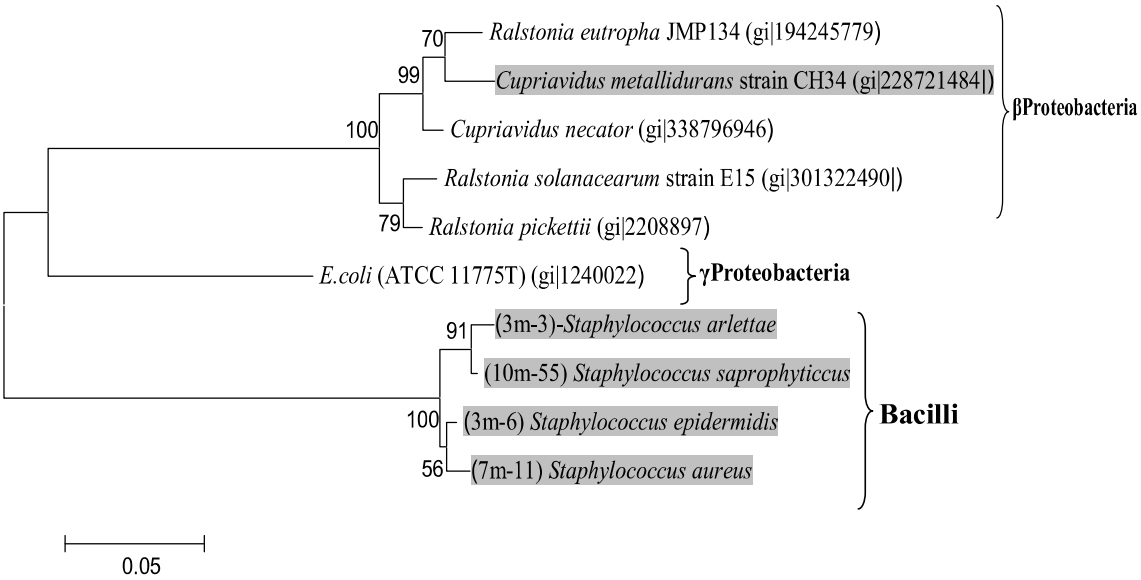

Figure 6 : Phylogenetic relationship between the *staphylococci* species, *C. metallidurans* and related bacteria. The phylogenetic tree was constructed using 16S rRNA genes from databases with MEGA version 4 (Tamura *et al.,* 2007).

Relation phylogénétique entre les Staphylococci, C. metallidurans, *et des bactéries apparentées. L'arbre phylogénétique a été construit en utilisant les gènes de l'ARNr 16S issus des banques de données. Le logiciel MEGA4 (Tamura et al., 2007) a été utilisé par la méthode du Neighbor-joining*

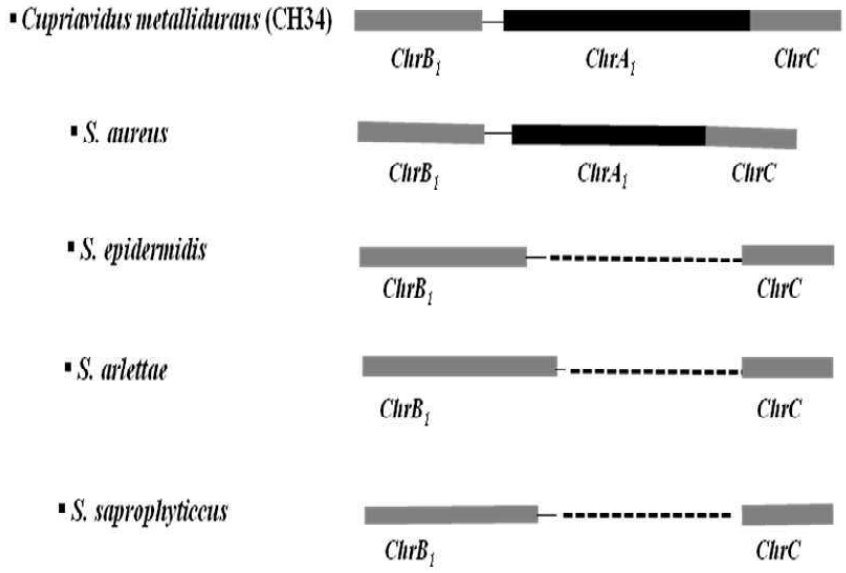

Figure 7 : Schematic representation of the chr determinants from different *Staphylococci* and the pMOL28 plasmid from CH34, showing the chromium resistant genes (*ChrA1, ChrB1* and *ChrC*).

Représentation schématique du déterminant chr issu de différents Staphylococci *et du plasmide pMOL28 de CH34, montrant les gènes de résistance au chrome* ChrA1, ChrB1 *et* ChrC.

DISCUSSION

Sixty-six chromium resistant *staphylococcal* strains belonging to *S. epidermidis*, *S. arlettae*, *S. aureus* and *S. saprophyticus* were isolated from a FA dumping site polluted by chromium (Kouadjo and Zézé, 2011). These *Staphylococci* species were distributed at different depths (3m, 7m and 10m) within the FA dumping site. Genes $ChrA_1$, $ChrB_1$ and *Chr*C were reported to be involved in chromium resistance in *C. metallidurans* (Nies *et al.,* 1990 ; Juhnke *et al.,* 2002). In order to see whether or not the chromium resistant *Staphylococci* species harbored these genes, in a first approach slot blot hybridizations were performed on genomic DNAs obtained from the 66 strains. The three genes amplified from the positive control *C. metallidurans* were used as probes. Using this approach, it was shown that gene $ChrB_1$ was present in all strains. Most of the strains harbored gene *ChrC* except a few of them. However the gene $ChrA_1$ was detected only in the genome of *S. aureus* but not in the genomes of *S. saprophyticus*, *S. epidermidis* and *S. arlettae*. Slot blot hybridization had been previously used for the detection of metal resistance genes in sulfate reducing bacteria (Diels and Mergeay, 1990 ; Naz *et al.,* 2005). However it was important to confirm these results. These results showed presumptive evidence of the presence of plasmid pMOL28 determinant that confer resistance to chromate in the genomes of the *staphylococci* species isolated from the fly ash dumping site. Another well-known technology for detecting metal resistance determinants is PCR amplification (Naz *et al*., 2005). We used this technique to confirm the results obtained in slot blot hybridization for genes $ChrB_1$ and $ChrA_1$. The primers used for this purpose allowed the amplification of gene $ChrA_1$ only in the genome of *S. aureus* confirming the result in slot blot hybridization. Southern blotting of digested genomic DNAs from the four species with $ChrA_1$ amplified from *C. metallidurans* also confirmed that only *S. aureus* harbored this gene. Moreover, the primers designed for PCR detection of gene $ChrB_1$ allowed the detection of this gene at variable sizes in the four species confirming its presence as revealed by slot blot hybridization. This study is the first report of the presence of chromium resistance determinants belonging to plasmid pMOL28 belonging to *C. metallidurans* in the genome of native *staphylococci* species. The phylogenetic analysis of 16S rRNA genes from *C.*

metallidurans and those from the *Staphylococci* species confirmed that these bacteria belong to two different lineages. *C. metallidurans* is a Gram-negative bacteria belonging to the Ralstonia lineage of the ß-*Proteobacteria* (Schmidt and Schlegel, 1994) while the *Staphylococci* species are Gram positive and belong to the Bacilli lineage. Meanwhile, it is not surprising that the plasmid pMOL28 which initially belongs to *C. metallidurans* was found in bacteria belonging to a divergent lineage. Transfer of metal resistance determinants have already been observed between Gram negative and Gram positive bacteria (Abou-Shanab, 2007). Due to the fact that determinants $ChrA_1$, $ChrB_1$ and *ChrC* are located on a plasmid, their transfer may have occurred by horizontal transfer within the *Staphylococci* genomes as previously shown in other microorganisms (Barkay *et al.,* 1985, Bogdanova *et al.,* 1988). The presence of $ChrB_1$ in different sizes may demonstrate that this gene may be distributed in different families within the *staphylococcal* community. However gene $ChrA_1$ which was found only in *S. aureus* may have been lost by transposition by the other *Staphylococci* species. Gene *ChrC* was shown to be present except in a few strains. All together, one might hypothesize that plasmid pMOL28 is present within the staphylococcal community in the dumping site in different conformations (Figure 7). As can be observed in the model proposed, except *S. aureus*, the three other species do not harbor ChA_1. If these species resist chromate toxicity as shown previously (Kouadjo and Zézé, 2011) on the basis of pMOL28 genetic functioning, does $ChrA_1$ play an important role ? It was shown that chromate resistance could be conferred even if the functioning of the $ChrA_1$ efflux pump was not effective in that the counterpart gene $ChrA_2$ which is chromosomal can play the same role (Juhnke *et al.,* 2002). The functioning of *chr* determinants in conferring resistance to chromium toxicity is a very complex mechanism (Ramý□rez-Dý□az *et al.,* 2008). It means that a thorough investigation is needed in order to understand the exact role of plasmid pMOL28 in chromium tolerance in the *Staphylococci* species.

ACKNOWLEDGEMENTS

The authors are grateful to the Institute of Microbial Biotechnology and Metagenomics (IMBM), Department of Biotechnology, University of Western Cape and its Director Professor Don Conwan for hosting Ms Kouadjo for her PhD scholarship.

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