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BROAD-RANGE BACTERIOCINS OF INDIGENOUS PSEUDOMONADS: *IN VIVO* MANIPULATION AND MOLECULAR CHARACTERIZATION

SHEIKH AJAZ RASOOL AND MUHAMMAD KHURRAM ZAKI KHAN
Department of Microbiology, University of Karachi, Karachi-75270. Pakistan

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Indigenously isolated clinical *Pseudomonas aeruginosa* were screened for bacteriocin production against *E. coli* AB712, *Staphylococcus aureus* ATCC 25923, *Agrobacter tumifaciens, Erwinia* AC 4075 and *Xanthomonas*. 44% of the isolates were found bacteriocinogenic against one or more indicator strains. The location (*viz.* chromosomal or extra-chromosomal) of the bacteriocin genes was determined by curing the representative producing cultures. 42% of the cured, producing pseudomonads lost bacteriocinogenic potential, thereby indicating non-chromosomal location. Plasmid mediated *in vivo* gene transfer (using *E. coli* AB712 and *E.coli* 40 as the recipient strains) studies gave stable exconjugants (10 out of 20 matings) which acquired the drug resistance marker; 5 of the stable exconjugants also acquired the bacteriocin genes against one or two indicator strains. One non-producing pseudomonas strain showed mitomycin C-mediated bacteriocin production. This protein was extracted and subjected to sodium dedecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and found to be of 50 ± 5 Kd molecular weight.

Key words: Pseudomonads, Bacteriocins, Broad-range plasmids, *In vivo* gene transfer, SDS-PAGE.

Introduction

Bacterial chromosome comprises a major linkage group containing most of the genes that control the cellular functions [1]. Many bacteria however, may harbor additional linkage groups i.e., plasmids are small autoreplicative, dispensable and physically separated from the chromosome [2]. Most of the plasmids have been discovered by virtue of some recognizable functions [3]. The bacteriocinogenic factors control the synthesis of protein antibiotics which kill other sensitive strains [4]. Protein antibiotic like substances (bacteriocins) are also produced by certain strains of pseudomonas [5]. Synthesis of such bacteriocins is controlled by the determinants located in the proper chromosome [6].

Pseudomonads constitute an interesting model for genetical studies. Therefore, bacteriocinogenic clinical pseudomonads were selected for the present studies which include: screening of the isolates for bacteriocinogenic potential against representative plant and human pathogens; determination of antibiotic sensitivity pattern of the bacteriocinogenic pseudomonads in order to select appropriate markers for plasmid conjugation studies; determination of the location of bacteriocinogenic determinants (for this purpose, acridine orange-mediated plasmid curing and plasmid-mediated conjugation were performed whereby bacteriocinogenic pseudomonads were used as donor and *E. coli* AB712 and *E.coli* BU40 as the recipients); and physico-chemical characterization of the bacteriocins from MMC-induced and uninduced pseudomonads that included ethanol extraction, followed by SDS-PAGE.

Materials and Methods

Bacterial strains. Pseudomonas *(Pseudomonas aeruginosa)* isolates were collected from different clinical laboratories of Karachi. These cultures were identified on the bases of cultural and biochemical characteristics [7]. The sensitive strains used in these studies are listed in Table 1.

TABLE 1. SENSITIVE BACTERIAL STRAINS.

1.	*E. coli* AB712
2.	*E. coli* BU40 *
3.	*Staphylococcus aureus* ATCC 25923
4.	*Xanthomonas*
5.	*Erwinia* AC 4075
6.	*Agrobacterium tumefaciens*

* = This train was not used as the sensitive culture.

Bacteriocin assay. Stab and overlay method (with some modifications) of Cooper and James [8] was followed for this purpose. Two parameters were followed for the determination of the location of the bacteriocinogenic determinants *viz.* acridine orange-mediated curing of plasmids of the producing strains (as per Hirota; [9] and *in vivo* gene tranfers (Plasmid-mediated conjugation) that were perfromed with *E.coli* AB712 and *E.coli* BU40 as the recipients [10]. Priorly, antibiotic sensitivity patterns of the representative producing pseudomonads were determined for selecting the suitable resistance markers. Stability of the transferred markers in the transcojugants was checked by replica plating. The suitable transconjugants were further checked for the transfer of the bacteriocinogenic determinants [8]. For characterization of the bacteriocins, 3 representative producing strains were induced with mitomycin C (with a final concentration of 1 $\mu g/ml^{-1}$) and the mothod of protein precipitation of Abbasi *et al.* [11] was followed. Molecular weights of the precipitates was determined by SDS-PAGE [12].

Results and Discussion

Figure 1 comprises of bar diagram indicating the Bacteriocinogenic activity of pseudomonads against 5 cultures. Table2 indicates stability of the transcojugants and the genotypes of the recipient strains. Accordingly, only 5 out of 13 transconjugants were found stable. Table 3 presents the drug resistance markers acquired by the 5 stable transconjugants. Table 4 depicts that the transferred bacteriocinogenic markers (in *E. coli* AB712 and *E. coli* BU40) are not effective against *Staph. aureus* but are effective against *Xanthomonas* and *Erwinia*. Figure 2 shows the SDS-PAGE of ethanol precipitated crude extracts (bacteriocins) of some of the pseudomonads, whereby, one of the non-bacteriocinogenic pseudomonads was depressed; while the other two cultures remained uninduced. The molecular weight of the proteins was found to be 50 ± 5 Kd.

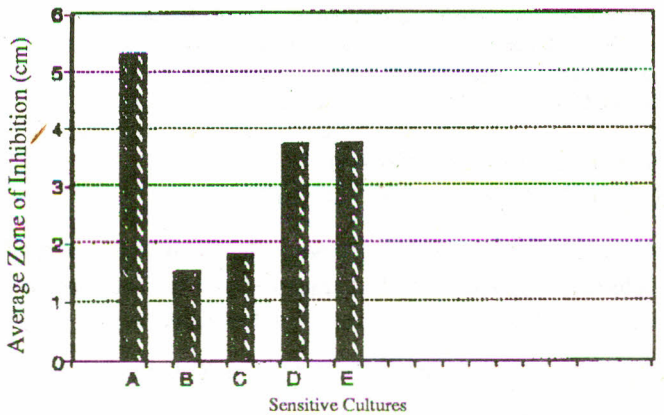

Fig. 1. Bacteriocinogenic activity of pseudomonads against 5 bacterial cultures.

X-axis: Zone of inhibition in cm; Y-axis: Sensitive bacterial strains. (A) *Staph. aureus* ATCC 25923. (B) *E. coli* AB 712. (C) *Agrobacterium tumefaciens*. (D) *Xanthomonas*. (E) *Erwinia* AC 4075.

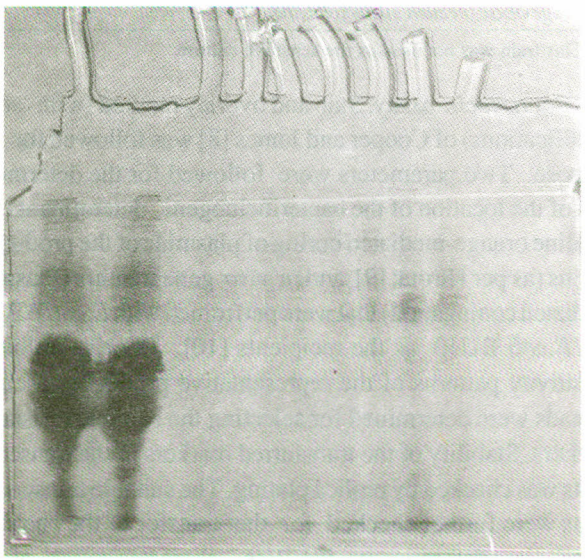

Fig. 2. SDS-PAGE of ethanol precipitated crude extracts of the bacteriocins of some of the clinical pseudomonas isolates (uninduced and induced by MMC).

Key from right to left: (1) Plain LBA, (2) Uninduced KM-5, (3) Induced KM-5, (4) Uninduced KM-14, (5). Induced KM-14, (6) Uninduced KM-16, (7) Induced KM-18, (8&9) Standard protein markers.

44% of the pseudomonas isolates were found bacteriocinogenic against one or more target cultures (2 human pathogens and 3 plant pathogens) Vidaver *et al.* [13] also reported a higher percentage of phytopathogenic bacteriocinogenic cultures among different species of pseudomonas.The extent of antagonism by the presently studied pseudomonads was appreciably more than the earlier reports [13]. There may be a number of reasons for the enhanced antagonistic effect e.g. strains carrying *rfa* or *tol* AB mutations may produce larger zone in plate tests [14]; the composition of the medium used, specially concentrations of different salts (e.g. 1 ml/lit of 0.002M $CaCl_2$ was found to be optimum in our studies) markedly effect the bacteriocin production and its binding ability with the receptors of the sensitive bacteria [15]. The present findings do not support the present concept whereby bacteriocins are effective only against closely related organisms [14,16]. The results have also revealed that the bacteriocinogenic determinants in 46% (for the plant pathogens) of the presently studied pseudomonads are located on the plasmids (based on plasmid conjugation experiments). These findings deviate from the concept that the bacteriocinogenic determinants in pseudomonads [6] and in a strain of *Klebsiella pneumoniae* [17] are located on the proper chromosome.

TABLE 2. STABILITY OF TRANSCONJUGANTS AS PROVED BY REPLICA PLATING ON MINIMAL MEDIUM M9 AND TWO DIFFERENT SETS OF ANTIBIOTICS TO ELEMINATE DONOR AND RECIPIENT.

Conjugation sets			
Donor	Recipient		Stability of
pseudomonads	*E.coli* AB712	*E.coli* BU40	exconjugants
KM-01	"	"	NS
KM-08	"	"	NS
KM-10	"	"	NS
KM-12	"	"	NS
KM-14	"	"	S
KM-15	"	"	NS
KM-17	"	"	NS
KM-18	"	"	S
KM-20	"	"	S
KM-23	"	"	S
KM-24	"	"	NS
KM-25	"	"	S
KM-26	"	"	NS

Key : NS = Not stable, S = Stable.

Genotype of the Recipient Strains: *E.coli* AB712 : F⁻ lac⁻ leu⁻ thr⁻ thi⁻ pro⁻ Sm^r 500 T6^s *E.coli* BU40 : F- ~Δ pro lac trp⁻ Sm^r 30

TABLE 3. DETERMINATION OF MARKERS ACQUIRED BY THE TRANSCONJUGANTS.

Cultures	Control 1	LB + *Sm 50,500	LB + Cm50	LB + Tc50	Minimal medium	Control 2
KM-14*	+	–	+	–	+	+
x *E. coli* AB712**	+	+	–	–	–	+
x *E. coli B*U40	+	–	–	–	–	+
Exconjugant	+	+	+	–	–	+
KM-18*	+	–	+	+	+	+
x *E.coli* AB712**	+	+	–	–	–	+
x *E. coli* BU 40	+	–	–	–	–	+
Exconjugant	+	+	+	+	–	+
KM-20*	+	–	+	–	+	+
x *E. coli* AB712**	+	+	–	–	–	+
x *E. coli* BU40	+	–	–	–	–	+
Exconjugant	+	+	+	–	–	+
KM-23*	+	–	+	+	+	+
x *E. coli* AB712**	+	+	–	–	–	+
x *E. coli* BU40	+	–	–	–	–	+
Exconjugant	+	+	+	+	–	+
KM-25*	+	–	+	+	+	+
x *E. coli* AB712**	+	+	–	–	–	+
x *E. coli* BU40	+	–	–	–	–	+
Exconjugant	+	+	+	+	–	+

* = Donor (*Pseudomonas* culture). ** = Recipient (*E. coli* culture). + = Growth. – = No growth. LB = Luria basal agar. Sm 500 = Streptomycin 500µg/ml. Sm 50 = Streptomycin 50µg/ml. Cm 50 = Chloramphenicol 50µg/ml. Tc 50 = Tetracycline 50µg/ml.

TABLE 4. DETERMINATION OF TRANSFER OF PSEUDOMONAD BACTERIOCINOGENIC FACTOR IN STABLE EXCONJUGANTS OF *E.* coli.

Pseudomonas isolates showing stable transconjugants	Zone of inhibition (cms) given by exconjugants (cultures of *E. coli*) against		
	Staph. aureus ATCC 25923	*Xanthomonas*	*Erwinia* AC4075
KM-14	–	–	+ (C/1.2)*
KM-18	–	+ (D)**	+ (C/0.4)*
KM-20	–	+ (D)*	–
KM-23	–	–	+ (C/2.0)*,**
KM-25	–	–	+ (C/1.0)**

* = Zone of inhibition by *E. coli* AB712 exconjugants. ** = Zone of inhibition by *E. coli* BU40 exconjugants. + = Bacteriocin produced. – = Bacteriocin not produced. (D) = Diffused zones. (C) = Clear zones. ( ) = Average inhibition zones (cms).

Similarly, the existence of broad-range plasmids (alongwith the bacteriocinogenic determinants) in Pseudomonads (as revealed in the plasmid gene transfer to the distant recipient like *E.coli* AB712 and *E. coli BU40*) are also being reported here. Analogous results have appeared in a recent study on the successful transfer of Ery^r factor from *P. aeruginosa* to *E. coli* BU40 [18]. Physico-chemical characterization of the bacteriocins by three representative pseudomonads (one non-producing and 2 producing) have shown that only the non-producing isolate could be induced resulting in a 50 ± 5 Kd protein. Earlier [19], MMC has also shown to increase the colicin titers of the colicinogenic cultures. It apppears that not all the bacteriocinogenic determinants of pseudomonads are inducible by MMC as has been demonstrated earlier [17]. The molecular weight of the presently studied bacteriocins falls within the earlier reported ranges (17-90 Kd) [20]. The findings of the present work seem to be interesting and formulate a good basis for further studies on the practical applications of these bacteriocins.

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