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ISOLATION AND BIOCHEMICAL CHARACTERIZATION OF MUTACIN VSM43 ISOLATED FROM HUMAN ORAL *STREPTOCOCCUS MUTANS* VSM43

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ABSTRACT

Bacteriocins produced by mutans streptococci are known as mutacins. *Streptococcus mutans* VSM43 isolated from human clinical oral cavity was screened for the production of mutacin. It can inhibit the growth of other mutans streptococci, many other gram-positives and some gram-negative bacteria. Average size of the inhibitory zone ranged between 12-20mm. The inhibitory activity could not be related to organic acids, bacteriophages and hydrogen peroxide. MutacinVSM43 was protease sensitive, remained active within a pH range of 2-8, and lost activity after heating at 100ºC for 30 min. MutacinVSM43 was dialyzable through dialysis membrane (pore size 12,000Da). A titre of 1280 arbitrary/activity units per ml (AU/mL) was shown against *Staphylococcus aureus*AB211. Lacuna frequency percentage (LF%) against *Streptococcus mutans*VSMD and *Staphylococcus aureus* AB211 was 37% and 49% respectively. We are convinced mutacinVSM43 may be a parallel candidate for use against dental caries.

INTRODUCTION

Recent years have witnessed a growing interest in a new approach for dental caries control, which is based on exploiting the ability of certain bacteria to produce antibiotics called bacteriocin-like inhibitory substances (BLIS) with promising bioactivity for mutans streptococci. Clinical mutans streptococci have been reported to produce antimicrobial activity hence the term mutacin has been proposed to describe these antimicrobial substances (Hamada and Ooshima 1975a). The mutacin activity of mutans streptococci has been determined on agar plates while, only a few strains of *S. mutans* have been reported to produce cell-free mutacin in liquid cultures (Delisle, 1986; Parrot *et al.,* 1989). Mutacin production was affected by conditions such as composition of the culture medium, incubation time and pH. Tagg and Russell (1981) found maximum production on tryptic soy agar having 1% neopeptone. Paul and Slade (1975) reported a non-diffusible bacteriocin from *S. mutans* GS5 (serotype c) with a molecular mass of >20KDa while, Hillman *et al*. (1984) described a serotype c strain of *S. mutans* that produced a mutacin with a molecular mass <1KDa. Mutacin-producing strain *S. sorbinus* MT6223 clearly inhibited colonization on tooth surfaces by *S. mutans* MT6222 (Kitamura *et al*. 1989). The mutacins A, B, C, D, I, K, L, M, and nisins A and Z were found active against all the enterococci tested (Morency *et al*, 2001).

MATERIAL AND METHODS

Strain, media and growth conditions:

*Streptococcus mutans*VSM43 (isolated from human oral cavity) was tested for mutacin activity. Cells were grown in brain heart infusion (BHI) broth or agar at 37ºC without aeration for 24h. Sensitive/indicator strains used include both gram-positive and gram-negative bacteria, procured from different pathological laboratories of the cosmopolitan city of Karachi. Producer as

well as sensitive cultures were maintained in small vials by growing them in 3mL of BHI broth and overlaid with 2mL of 40% glycerol after 24h incubation.

Mutacin activity assays:

Following two methods were used to check mutacin activity

Stab-overlay method (SOM):

BHI agar plates were stabbed with *Streptococcus mutans*VSM43 and incubated at 37ºC for 24h. Next day plates were exposed to chloroform vapours for 20-30min to kill the bacteria. Plates were then overlaid with 3mL soft agar containing $2x10^8$ cells of indicator organism, re-incubated at 37°C for overnight to observe the clear zone around the producer culture (Rasool *et al*. 1996).

Agar-well diffusion method (AWDM):

*Streptococcus mutans*VSM43 was grown in brain heart infusion broth and incubated at 37°C for 24h. Next day cell free supernatant was obtained by centrifugation (10,000rpm, for 30min, 4°C). The cells were discarded and pH of the supernatant fluid was adjusted to 7.0 with 1N NaOH. Plates were overlaid with 3mL soft agar containing $2x10^8$ cells of indicator organism. Wells (5mm diameter) were cut and 100µl of cell free neutralized supernatant fluid (CFNS) of the producer organism was poured into each well. Next day zone of inhibition of the indicator strain around the well was measured. Mutacin titer was determined by the serial dilution assay and was expressed as activity unit/mL. One activity unit (AU) of bacteriocin is defined as the reciprocal of the last serial dilution demonstrating inhibitory activity (Muriana and Klaenhammer, 1991).

Detection of lytic bacteriophages:

Reverse-side technique was applied, which prevents the contact of bacteriophages with the producer and the indicator strains. The procedure was same as stab-overlay test except that the indicator strain was poured on the face opposite to the one where the producing strain was grown, so that contact between indicator and producing strain was not possible (Parrot *et al*., 1990).

Detection of hydrogen peroxide and lactic acid production:

To rule out the inhibitory activity of the mutacinVSM43 by hydrogen peroxide or lactic acid production, the method of (Iqbal *et al*., 1999) was followed. For this 1.0mL suspension of crude mutacin was mixed with 1.0mL of 100mM catalase (4000units/mL) and was assayed by agar well diffusion assay. The controls included mutacin preparation (as positive control) and catalase 4000units/mL (as negative control). Similarly, potential inhibitory activity of mutacinVSM43 due to acid as a normal consequence of metabolic activities was also ruled out by growing *S. mutans*VSM43 in a buffered medium and then after an overnight incubation bacteriocin-mediated antagonism was examined.

Thermostability and enzyme treatment:

To determine the heat sensitivity of the mutacinVSM43, 24h old stabbed cultures were exposed to 60ºC, 80ºC and 100ºC in hot oven and the activity was checked by stab-overlay method using *S. mutans*VSMD as sensitive culture. Similarly, heat-treated cell-free neutralized supernatant (CFNS) of mutacinVSM43 was also tested by agar-well diffusion approach. Effect of pH values and different enzymes on stabbed cultures and CFNS of mutacinVSM43 was also checked (Parrot *et al*., 1989 and Iqbal *et al*., 2001).

Molecular mass estimation:

The diffusibility of mutacinVSM43 through dialyzable membrane (pore size12,000Da) was checked to estimate its molecular weight. For this, 24h old stabbed culture was covered with

sterile piece of dialyzable membrane and then overlaid with 5mL soft agar. After 24h incubation results were recorded. (Parrot *et al.,* 1989).

Lacuna assay:

Lacuna assay (as per Ozeki *et al*., 1962) was performed to determine the frequency of mutacin producing *S. mutans*VSM43 out of a whole population. *S. mutans* VSM43 was grown in BHI broth under optimum conditions. Serial dilutions (10-fold) were made in 0.9mL BHI broth and then 0.1mL was poured into the plates and incubated at 37°C for 24h to get isolated colonies. Assay mixture contained 3mL of soft agar containing 1mL of exponentially growing indicator cultures i.e. *S mutans* VSMD or *S aureus* AB211. Plates were re-incubated at 37°C for overnight for lacuna forming cells counts.

RESULTS AND DISCUSSION

Production of antagonistic substances is an important factor in microbial ecology. Among many different substances known to play a role in bacterial interactions, bacteriocins are the most specific yet efficient antagonists.

Inhibitory spectra:

Mutacin production was determined by stab-overlay assay and agar-well diffusion assays (Figs.1 and 2). Accordingly, the mutacinVSM43 activity was demonstrated by both assays against different gram-negative and gram-positive indicator strains. Infact, this observation is in relevance to broad range antagonistic activity but (surprisingly) the bioactivity against streptococcal indicator strains could be demonstrated only on solid medium and not in cell free neutralized supernatant (CFNS). However, we did get activity in culture supernatant against other intergeneric strains such as *Staphylococcus aureus*, *Neisseria meningitidis*, *Xanthomonas maltophila* etc. (Table 1). The extent of bioactivity (inhibition zones) varied from strain to strain (sensitive strains). Our results are in close agreement with those of Hamada and Ooshima (1975a,b), Parrot *et al.* (1990), and Balakrishnan *et al.* (2002), who reported that the mutacin activity of mutans streptococci was well-recognized on agar plates. However, only a few strains of *S. mutans* have been reported to produce cell-free mutacin in liquid culture. It appears that mutacin release needs a solid substrate. An interesting observation is the sensitivity of *Neisseria meningitidis*. This genus has often been reported to be sensitive to mutacins (Tagg and Ruseell, 1981; Fabio *et al*., 1987). The titre of crude preparations of *S. mutans* VSM43, was 1280AU/mL against *S. aureus* AB211 (Fig. 3).

Inhibition due to bacteriocin molecules or other agents?

In order to rule out the possibility of the bioactivity due to non-mutacin agent, tests were performed for the activity due to lytic phages, hydrogen peroxide or lactic acid. Bacteriocins, unlike bacteriophages do not carry the genetic determinants necessary for self-replication within susceptible organisms. Thus, when a block from zone of inhibition was cut and emulsified in sterilized BHI broth, we did not get any inhibition zone thereby suggesting that the inhibitory molecules were not bacteriophages rather they may be bacteriocin like molecules. Only bacteriophages can be propagated on cultures of the indicator strains. Similarly, inhibition due to hydrogen peroxide and lactic acid could not be established, as earlier studied by Parrot *et al.* (1989 and 1990).

Table 1
Mutacin VSM43 activity of *S. mutans* VSM43 against gram-positive, gram-negative bacteria and yeast cells

Indicator organisms	*S. mutans* VSM43			
	SOM		AWDM	
Gram-positive bacteria	**A**	**B**	**A**	**B**
Bacillus subtilis.	0/5	0	0/5	0
Clostridium perfringens	0/1	0	0/1	0
Corynebacterium diphtheriae	1/1	18	1/1	19
Lactobacillus acidophilus	4/6	12	4/6	10
Micrococcus lysodiecticus	7/10	20	7/10	25
Listeria monocytogenes	4/6	12	4/6	10
Staphylococcus aureus	40/50	20	40/50	25
Staphylococcus epidermidis	4/12	10	4/12	20
Staphylococcus saprophyticus	1/1	15	1/1	19
Streptococcus agalactiae	4/5	13	0/5	0
Streptococcus equi	10/15	13	0/15	0
Enterococcus faecalis	6/10	14	0/10	0
Enterococcus faecium	5/10	10	0/10	0
Streptococcus mutans	30/30	15	0/30	0
Streptococcus pneumoniae	16/26	15	0/26	0
Streptococcus pyogenes	10/30	15	0/30	0
Streptococcus sanguis	4/4	15	0/4	0
Gram-negative bacteria				
Agarobacterium tumefaciens	0/1	0	0/1	0
Escherichia coli AB712	0/1	0	0/1	0
Escherichia coli BU40	0/1	0	0/1	0
Escherichia coli 5014	0/1	0	0/1	0
Escherichia coli WT	0/15	0	0/15	0
Klebsiella pneumoniae	2/12	2	0/12	0
Neisseria meningitidis	2/4	19	2/4	20
Proteus vulgaris	0/2	0	0/2	0
Pseudomonas aeruginosa	0/17	0	0/17	0
Salmonella typhi	0/5	0	0/5	0
Salmonella paratyphi A	0/5	0	0/5	0
Salmonella paratyphi B	0/5	0	0/5	0
Shigella dysenteriae	0/1	0	0/1	0
Xanthomonas maltophila	1/1	10	1/1	20
Yeasts				
Candida albicans	0/8	0	0/8	0
Saccharomyces cerevisiae	0/2	0	0/2	0

A, Strains sensitive/Strains tested; B, Average zone size (mm); 0, No zone of inhibition.

Physico-chemical characterization:

It was done (by both stab-overlay and agar-well diffusion assay) in order to check the effect of different physical and chemical agents on mutacinVSM43 against two indicator cultures i.e. *S. mutans* VSMD and *S. aureus* AB211 (Table 2). As mentioned earlier, CFNS demonstrated bioactivity against *S. aureus* AB211 only but not against *S. mutans* VSMD, while stabbed cultures showed inhibitory activity against both *S. aureus* AB211 and *S. mutans* VSMD cells.

MutacinVSM43 remained stable at 100°C (30min) whereas mutacin MT6223 resisted 100°C for 20min (according to Loyola-Rodriguez *et al*., 1992). Mutacin MT6223 was also found stable after 2 month incubation at 4°C. Parrot *et al*. (1990) reported that bactericidal activity of mutacin NY257-S was lost after 1 week on agar plates, while mutacins C67-1 and T8 retained bioactivity for 1 month and mutacin NY266 sustained the bioactivity even after 2 months. Mutacin VSM43 was also found stable at 2-8 pH range. Rasool *et al*. (1996) also reported that streptococcin Sam 51 and Sam 53 were not affected through 2-8 pH range treatments.

Mutacin activity was completely lost by protease (1mg/mL), while lipolytic and glycolytic enzymes had no effect on it. Loyola-Rodriguez *et al.* (1992) reported that mutacin from *S. sorbinus* was partially inhibited by α-chymotrypsin while completely inactivated by papain or ficin digestion.

Lacuna frequency:

The percentage of cells producing bacteriocin out of the whole population is determined by lacuna assay (Ozeki *et al.,* 1962). Lacunae are the clear haloes produced in the lawn of the indicator bacteria by the bacteriocins released from the individuals cells. In our results lacuna percentage in *S. mutans* VSM43 was 37% against *S. mutans* VSMD and 49% against *S. aureus* AB211. Infact, lacuna percentage assay has been valuable against different sensitive cultures.

Broad bioactivity spectrum of mutacinVSM43 and its resistance to high temperature and pH, make it possible candidate for future parallel therapeutics. However, further investigations are warranted for this purpose.

Table 2

Effect of physio-chemical treatments on mutacin VSM43

Treatments on mutacinVSM43		
Method	**SOM**	**AWDM**
Sensitive culture	***S. mutans* VSM43**	***S. aureus* AB211**
Temperature		
60ºC for 30min	R	R
80ºC for 30min	R	R
100º C for 30min	S	S
pH		
2-8	R	R
Enzymes		
Lipase	R	R
Lysozyme	R	R
Protease	S	S

Fig. 1: Stab-overlay method demonstrating mutacinVSM43 activity against *S. aureus* AB211.

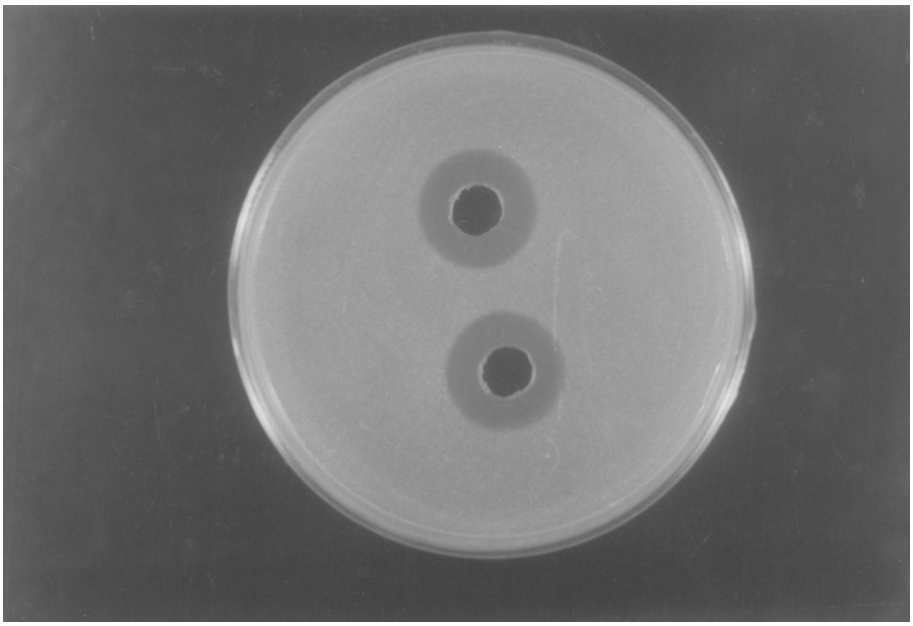

Fig. 2: Agar-well diffusion method demonstrating mutacinVSM43 bioactivity against *S. aureus* AB211.

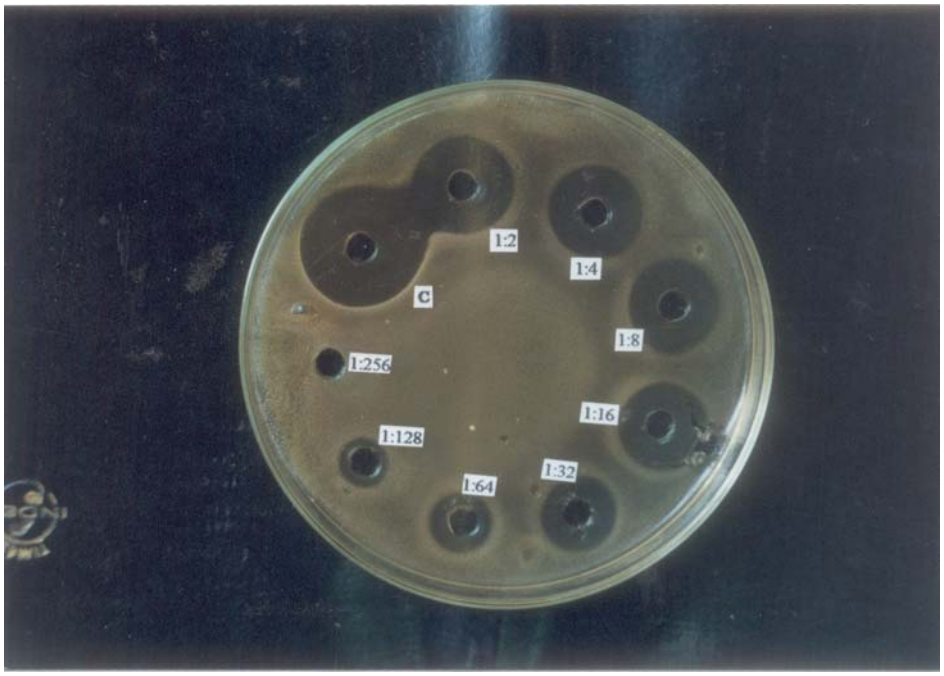

Fig. 3: Agar-well diffusion assay demonstrating mutacin bioactivity of *Streptococcus mutans* VSM43 in terms of arbitrary units per mL against *Staphylococcus aureus* AB211

$$\text{AU/mL} = \frac{\text{Reciprocal of the highest dilution} \times 1000}{\text{Volume of bacteriocin added}}$$

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