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Inhibitory efficacy of cyclo(L-leucyl-L-prolyl) from mangrove rhizosphere bacterium–*Bacillus amyloliquefaciens* (MMS-50) toward cariogenic properties of *Streptococcus mutans*

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Abstract

Since *Streptococcus mutans* is the principal etiologic agent causing dental caries, by encompassing an array of unique virulence traits, emerging treatment strategies that specifically target the virulence of this pathogen may be promising as alternative approaches compared to conventional antibiotic therapy. In this perspective, we investigated chloroform extract of cell-free culture supernatant from mangrove rhizosphere bacterium *Bacillus amyloliquefaciens* (MMS-50) in terms of anticariogenic properties of *S. mutans*, without suppressing its viability. Crude chloroform extract of MMS-50 was subjected to column and high performance liquid chromatographic techniques to obtain the active fraction (AF), and MMS-50 AF was used for all further assays. GC–MS and FT-IR were carried out to identify the major components present in MMS-50 AF. Comparative gene expression analysis of some biofilm-forming and virulence genes (*vic*R, *com*DE, *gtf*C, and *gbp*B) was done by real-time PCR. Cyclo(L-leucyl-L-prolyl) was found to be the chief compound in MMS-50 AF responsible for bioactivity. The minimum and maximum inhibitory concentrations of MMS-50 AF against *S. mutans* were found to be 100 and 250 μg/mL, respectively. Anti-virulence assays performed using below-sub-MIC levels of MMS-50 AF (30 μg/mL) resulted in significant reduction in adherence (68%), acid production, acid tolerance, glucan synthesis (32%), biofilm formation (53.5%) and cell surface hydrophobicity, all devoid of affecting its viability. The micrographs of CLSM and SEM further confirmed the antibiofilm and anti-virulence efficacies of MMS-50 AF. Expression data showed significant reduction in expression of all studied virulence genes. Thus, the current study unveils the anticariogenic potential of cyclo(L-leucyl-L-prolyl) from *B. amyloliquefaciens*, as well as its suitability as a novel and alternative anticariogenic agent against dental caries.

Keywords: Mangrove rhizosphere bacteria; Glucosyltransferases; *Bacillus amyloliquefaciens*; Anticariogenic agent

1. Introduction

Globally, dental caries is one among the three major non-communicable infectious diseases in humans, and is primarily instigated by irreversible dental plaque biofilm formation leading to progressive tooth decay [14]. *Streptococcus mutans*, a Gram-positive, facultative, anaerobic endogenous oral bacterium, has long been recognized as one of the principal etiological agents of dental caries [37]. Though the ecological niche of oral biofilm encompasses more than 500 different species of microorganisms, the survival of *S. mutans* is unhampered due to its extreme aciduricity, acidogenicity and genetic transformability, as well as its ability to produce a wide range of bacteriocins [36]. Thereby, *S. mutans* eventually dissolves the hard crystalline structure of the teeth, resulting in carious lesions [26]. In addition to the complex pathogenicity of *S. mutans* in causing dental caries, it was also reported to damage endothelial tissues by means of its attachment to platelet–fibrin matrices of the bloodstream entering via bruises in the oral cavity [20].

Unlike most infectious agents that exhibit classic virulence factors like endotoxins, the unique pathogenic traits of *S.*

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mutans are its ability to form recalcitrant biofilms, intense production of acid (acidogenicity) through carbohydrate metabolism and its capacity to survive at low pH, i.e. acid tolerance (aciduricity) and other environmental stresses. Another essential virulence factor often documented in *S. mutans* is its ability to produce glucosyltransferases (GTFs), which synthesize both intracellular polysaccharides (IPSs) and extracellular polysaccharides (EPSs) from dietary carbohydrates. *S. mutans* synthesizes three different GTFs, namely GtfB, GtfC and GtfD encoded by the genes *gtf*B, *gtf*C and *gtf*D, respectively. Among the GTFs, GtfB and GtfD play a vital role in synthesizing water-insoluble (α-1, 3-glucosidic linkages) and water-soluble (α-1, 6-glucosidic linkages) glucans, respectively, while GtfC produces both the water-soluble and -insoluble glucans [8]. GtfB and GtfC are recognized as critical virulence factors, particularly for the initiation of dental caries. These polysaccharides are the essential constituents of the oral biofilm matrix, as they influence efficient colonization and subsequent development of biofilms on different surfaces within the oral cavity by serving as an extracellular carbon source and modulating permeability to water and nutrients [19]. The inimitable ability of *S. mutans* to produce acid and tolerate the acidic pH further favors its continual survival and colonization in the dental biofilm [22]. GTFs in combination with acids attribute to devastating cariogenic effects like localized dental decalcification, cavity formation and breakdown of a calcified tooth followed by dissociation of tooth enamel [12]. Therefore, the current study was highly focused on the inhibition of various virulence factors that ascribe the cariogenic properties of *S. mutans*, which could possibly be a convincing target for combating or preventing biofilm-related dental caries infection.

Earlier, the traditional approach that sought to prevent biofilm formation in vivo involved local administration of biocides [7]. Nevertheless, the biofilms formed by pathogenic bacteria often persist, even in the presence of large doses of traditional chemotherapeutic agents [9]. Subsequently, several approaches were proposed, and were expected in the recent past to be proficient in directly preventing or eradicating bacterial biofilms. In their midst, anti-virulence therapy has been emerging as an alternative approach to the emergence of antimicrobial-resistant bacterial communities owing to the indiscriminate use of current generation therapeutic agents.

Mangrove rhizosphere bacteria remain an underexploited and prolific source of significant pharmaceutical thrust. The quest for microbial consortia with therapeutic properties continues to receive great attention, as researchers investigate marine microbes for an inclusive stretch of biological activities – antimicrobial, antiinflammatory, antibiofilm and antitumor. The probability of finding novel bioactive compounds is highly promising concerning microbiota associated with the marine (mangrove) ecosystem compared to microbes from the terrestrial environment [30]. Many studies in the recent past have clearly demonstrated the antipathogenic, anti-biofilm and anti-quorum-sensing properties of these marine bacteria against Gram-positive and -negative pathogens [10,23,34]. The comprehensive pharmaceutical significance of marine microbiota has been extended to several pathogens. The present study was intended to delineate the antibiofilm and anticariogenic properties of a mangrove rhizosphere bacterium, namely *Bacillus amyloliquefaciens* (MMS-50), against the cariogenic *S. mutans*. Studies in the recent past have clearly demonstrated the proficiency of *B. amyloliquefaciens* as a biocontrol agent in suppressing soil-borne pathogens through secretion of an array of lipopeptides and root colonization [38,41].

2. Materials and methods

2.1. Bacterial strains, culture medium and growth conditions

In the present study, the strain *S. mutans* UA159 from thawed suspension stored as a glycerol stock (20%) in an ultra-low deep freezer (−80 °C) was used for streaking/inoculating brain heart infusion (BHI) agar/broth (HiMedia Laboratories, Mumbai, India) and incubated at 37 °C for 24 h under anaerobic condition consisting of 80% N_2, 10% CO_2 and 10% H_2.

The mangrove rhizosphere bacterium MMS-50 was isolated from the Karankadu mangroves of Palk Strait, Bay of Bengal, India. The geographic location was latitude 9° 36′ N and longitude 78° 83′ E. The MMS-50 was grown and maintained on Zobell marine agar/broth (ZMA/ZMB) (HiMedia Laboratories, Mumbai, India) plates at 27 °C. *Chromobacterium violaceum* ATCC 12742 was used as a quorum sensing (QS) biomonitor strain.

2.2. Bacterial extract preparation and purification of the inhibitory compound

The cell-free culture supernatant (CFCS) of MMS-50 was obtained by centrifugation (20,929× *g* for 10 min) of the culture grown in ZMB for 48 h at 27 °C. This CFCS was then filtered through a 0.2 μm membrane filter and extracted with an equal volume of different solvents (on a polarity basis) and the chloroform extract demonstrated better antibiofilm activity than other solvents. Further, the chloroform extract was evaporated to dryness under reduced pressure at room temperature to yield crude extract [10]. After filtration and evaporation, the crude extract was oven-dried at 55 °C. The obtained crude extract was weighed and then re-dissolved in chloroform (for purification)/sterilized MilliQ water (for bioassay) to give the desired concentration.

The chloroform extract was then chromatographed on a silica gel (Silica gel G-5, Sisco Research Laboratories, India) column (50 cm × 2 cm), with solvents based on their increasing polarity. The adsorbed compound was eluted with chloroform and acetone (50:50) under a flow rate of 1 mL min^{-1}. Fractions (20 mL each) were collected separately and tested for activity by bioassay. The active fractions were pooled and evaporated. The fractions showing antibiofilm activity were further purified by the same column chromatography system, and purity was confirmed by thin layer chromatography (TLC) and bioassay. The purified active fraction (AF) was oven-dried at 45 °C. The dried AF was dissolved in sterile distilled MilliQ water to obtain a final