Congo red-binding protein in rough-phenotype aggregatibacter actinomycetemcomitans is amyloid-like fiber

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Journal
Bulletin of Tokyo Dental College, 50(1): 23-29

URL
http://hdl.handle.net/10130/996
Congo Red-binding Protein in Rough-phenotype 

Aggregatibacter actinomycetemcomitans is Amyloid-like Fiber

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Received 16 January, 2009/Accepted for publication 20 February, 2009

Abstract

Aggregatibacter actinomycetemcomitans is a pathogen associated with chronic and aggressive periodontitis and extra-oral infections. Fresh isolates of A. actinomycetemcomitans are fimbriated, forming small, rough-phenotype colonies on agar plates and also form biofilms. Recently, it has been reported that amyloid fibers are abundant in natural biofilms, and Escherichia coli and Salmonella spp. produce amyloid fibers that contribute to biofilm formation. This has yet to be reported, however, in A. actinomycetemcomitans. Amyloid binds the Congo red (CR) dye. In this study, therefore, we investigated amyloid formation in A. actinomycetemcomitans using a detection of CR-binding colonies on CR agar plates and CR-binding assay. All rough-phenotype strains formed dark red colonies and smooth-phenotype strains formed white or opaque red colonies on CR agar plates. Compared with smooth-phenotype strains, rough-phenotype strains showed higher CR-binding activity. CR-binding of rough-phenotype strain AKR was not affected by protease digestion or heating, whereas smooth-phenotype strain 29523 showed a marked reduction in CR-binding after both types of treatment. AKR showed amyloid-positive staining with CR to produce yellow green birefringence under polarized light, whereas 29523 showed amyloid-negative staining. These findings indicate that the CR-binding component of rough-phenotype A. actinomycetemcomitans is an amyloid-like fiber.

Key words: Aggregatibacter actinomycetemcomitans—Congo red-binding—Amyloid

Introduction

Aggregatibacter actinomycetemcomitans is a gram-negative facultative anaerobe, and has been implicated in the etiology of chronic and aggressive periodontal disease. This organism produces a number of potential virulence factors, including endotoxin, fimbriae, leukotoxin, hemolysin and invasive factors. Fresh clinical isolates of A. actinomycetemcomitans...
produce fimbriae and form rough-phenotype colonies which adhere strongly to the surfaces of agar plates, and also form biofilms\(^ {25}\). The transformation from rough to smooth phenotype is associated with loss of fimbriae\(^ {11}\). These phenotypic changes are also associated with its ability to cause disease in an experimental model\(^ {24}\).

Congo red (CR) is a planar, hydrophobic, diazo dye that binds to lipids, lipoproteins and variety of amyloid proteins. The yellow green birefringence of CR-stained preparations under polarized light is indicative of the presence of amyloid fibers\(^ {3}\). Amyloid fibers are highly organized protein aggregates resistant to chemical or temperature denaturation and digestion by proteases\(^ {20}\) and are found in association with a variety of human diseases, including Alzheimer’s disease\(^ {6}\) and spongiform encephalopathies (prion diseases)\(^ {9}\). Bacteria such as *Escherichia coli* and *Salmonella* spp. produce extracellular amyloid, known as curli, to help create an extracellular matrix that enables surface adhesion and biofilm formation\(^ {1}\). The biofilms consist of an extracellular matrix, including polysaccharides, proteins and nucleic acids. Recently, it has been reported that amyloid proteins are abundant in natural biofilms, and a recent report suggested that up to 40% of the biomass in activated sludge consists of amyloid-like filaments\(^ {18,19}\). Therefore, *A. actinomycetemcomitans* may form amyloid-like fibers in biofilm.

The present study investigated amyloid-like fiber formation in rough and smooth phenotype *A. actinomycetemcomitans* strains by detection of CR-binding colonies on agar plates and CR-binding assay.

### Materials and Methods

#### 1. Bacterial strains and culture conditions

Nine *A. actinomycetemcomitans* strains including fresh clinical isolated strains AKR, AB55 (Table 1) were cultured in Trypticase soy agar (TSA) and Trypticase soy broth (TSB) (BBL Microbiology Systems, Cockeysville, MD, USA) at 37°C in 5% CO\(_2\) in air. TSB medium with or without the addition of the iron chelator 2,2-dipyridyl (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 0.2 mM was used to assess the effect of iron. *E. coli* HB101 was used as negative control for CR (Sigma-Aldrich)-binding activity.

#### 2. CR binding assay

CR-binding on CR agar plates was determined in *A. actinomycetemcomitans* cultured in TSA containing 0.003% CR at 37°C in 5% CO\(_2\) in air for 3 days, and the results were observed with a stereoscopic microscope. CR-binding activity was also determined by modification of the procedures described by Kay *et al.*\(^ {13}\). *A. actinomycetemcomitans* was cultured in TSB at 37°C in 5% CO\(_2\) in air for 3 days. After centrifugation at 9,000 × g for 10 min, precipitates were collected and subsequently resuspended in phosphate-buffered saline, pH 7.4, (PBS) to an OD\(_{600}\) of 1.0 (1 × 10\(^9\) cells/ml) with the Ultrospec 3100pro (GE Healthcare Biosciences, Piscataway, NJ, USA). Cells were incubated at 37°C for 30 min in the presence of 50 µg/ml CR. After centrifugation at 12,000 × g for 1 min, supernatant was collected and added to 2 ml PBS. Binding of the CR was determined by assaying absorbance of the residual dye in the supernatant at 480 nm. Amount of residual dye was then compared with the standard curve of the CR, and the amount of CR bound by the bacteria was

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Table 1 *A. actinomycetemcomitans* strains used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Colony phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y4</td>
<td>s</td>
<td>ATCC</td>
</tr>
<tr>
<td>29523</td>
<td>s</td>
<td>ATCC</td>
</tr>
<tr>
<td>29524</td>
<td>s</td>
<td>ATCC</td>
</tr>
<tr>
<td>JP2</td>
<td>s</td>
<td>Tsai(^ {20})</td>
</tr>
<tr>
<td>310-a</td>
<td>r</td>
<td>Harano(^ {6})</td>
</tr>
<tr>
<td>AB55</td>
<td>r</td>
<td>Takahashi(^ {25})</td>
</tr>
<tr>
<td>AKR</td>
<td>r</td>
<td>This study</td>
</tr>
<tr>
<td>137he</td>
<td>s</td>
<td>Kimizuka(^ {25})</td>
</tr>
<tr>
<td>146he</td>
<td>s</td>
<td>Kimizuka(^ {25})</td>
</tr>
</tbody>
</table>

r: rough, s: smooth, ATCC: American Type Culture Collection
3. CR binding to cells after various pretreatments

The effects of various pretreatments on CR-binding activity were determined. Whole cells \(1.0 \times 10^6\) cells suspended in 1 ml PBS were subjected to proteolytic digestion at 37°C for 30 min with the addition of protease K (Boehringer Mannheim, Mannheim, Germany) and trypsin (Sigma-Aldrich) to a final concentration of 50 \(\mu\)g/ml. Proteolysis was terminated by addition of 1 mM PMSF (Sigma-Aldrich) and the CR-binding assay was performed as described above. The effects of various temperatures on CR-binding ability were determined after incubation at 37°C, 60°C or 100°C. The effect of formic acid on CR-binding ability was also determined. Whole cells were treated in 100 \(\mu\)l 99% formic acid on ice for 10 min, after which the liquid was removed by evaporation in the Speed Vac. The pellet was resuspended in 1 ml PBS. The binding assay for Congo red was carried out as described above. Assays that served as 100% binding controls were carried out with each of the suspensions less the test substance. All the assays were performed in triplicate. The binding values of the treated cells with test substance were calculated as a percentage of the 100% binding controls. The Mann-Whitney U-test was used for comparison of CR-binding assay after various treatments.

4. Assessment of amyloid properties by microscopy

A test for yellow green birefringence was performed. Colonies of tested strains grown on TSA containing CR were recovered and resuspended in PBS, after which the suspensions were allowed to dry on a glass slide. Samples were embedded in Malinol mounting medium (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), covered with a cover glass and analyzed with an Olympus IX71 equipped with a polarizer (Olympus Co., Tokyo, Japan).

Results

1. Binding of Congo red

Tested strains grown on TSA agar plates containing CR were classified into three phenotypes: white type colonies: smooth, large, red center with lighter outer zone; opaque red type colonies: smooth, large, opaque red; dark red type colonies: rough, small, dark colonies. As shown in Fig. 1, rough-phenotype AKR formed dark red type colonies. In contrast, Y4 formed white type colonies. The 29523 formed opaque red type colonies. In the CR-binding assay, rough-phenotype AKR was strongly bound by CR (Table 2). The CR-binding activity of rough-phenotype AKR, 310a and AB55 was stronger than that of smooth-phenotype strains. White type colonies, including Y4, 137he and 146he, showed low binding activity. The CR-binding activities of rough-phenotype AKR and smooth-phenotype 29523 grown under iron limited conditions were almost the same levels as those under iron standard condition, respectively (Table 2), indicating that iron-limited growth did not affect CR-binding.

2. Effect of bacterial pretreatment on CR binding

The effect of various pretreatments on CR-binding activity was shown in Table 3. Heating at 100°C for 5 min and treatment of AKR cells with Proteinase K or trypsin showed no effect on CR-binding activity. Pretreatment
of AKR with formic acid significantly reduced CR-binding activity by 74.2% in comparison with the control value (p<0.01). Heating of 29523 cells at 100°C for 5 min resulted in a significant reduction in CR-binding compared with the controls (p<0.01). Heating of 29523 at 60°C for 30 min significantly reduced CR-binding activity by 31.5% in comparison with
the control value (p<0.01). Treatment of 29523 cells with Proteinase K or trypsin significantly reduced CR-binding activity (p<0.01).

3. Rough-phenotype strains exhibited yellow green birefringence

Yellow-green birefringence of CR-stained preparations under polarized light is indicative of the presence of amyloid fibers. Therefore, we also investigated yellow-green birefringence of CR-staining with a polarized microscope. Transmission microscopy revealed that AKR and 29523 exhibited CR-staining, whereas Y4 did not (Fig. 2a, c and e). Polarized microscopic analysis revealed that rough-phenotype AKR exhibited yellow green birefringence, whereas smooth-phenotype strains 29523 and Y4 did not (Fig. 2b, d and f).

Discussion

In this study, rough-phenotype AKR colonies showed green fluorescence in polarized light after staining with CR dye, suggesting that the CR-binding protein of the rough-phenotype
is an amyloid-like fiber. Amyloid fibers are 4–10-nm wide, unbranched filaments with a characteristic cross-β-sheet rich structure, and are resistant to chemical or temperature denaturation and digestion by proteinases. Pretreatment with formic acid disrupts the β-sheet structure of amyloids. In this study, treatment of rough-phenotype AKR whole cells with proteases or heating showed no effect on CR-binding, but pretreatment with formic acid inhibited CR-binding. Thus, it was confirmed that the CR binding activity of the rough-phenotype AKR was due to amyloid-like fiber. Opaque red type A. actinomycetemcomitans showed low CR-binding activity. However, the CR-binding assay and microscopic analysis under polarized light revealed that the CR-binding of the smooth-phenotype was due to a protein component other than amyloid. A. actinomycetemcomitans has been reported to express cell surface hemin and CR-binding protein.

Harrison et al. suggested that soluble folding intermediates were the key to cytotoxicity and disease development. Recent studies have identified functional amyloid fibers in bacteria and mammals. Curli are one of the best-characterized functional amyloids. Curli are highly aggressive surface fibers assembled by E. coli. They stimulate the host inflammatory response and contribute to persistence within the host, and are also required for formation of biofilms. Amyloid fibers are abundant in natural biofilms such as activated sludge, drinking water biofilm and sea water biofilm. The results of the present study suggest the involvement of amyloid in the organization of rough-phenotype colonies in A. actinomycetemcomitans.

In conclusion, the results of the present study indicate that the heat-stable, CR-binding component of rough-phenotype A. actinomycetemcomitans is an amyloid-like fiber.

Acknowledgements

This research was supported by Oral Health Science Center Grant HRC7 from Tokyo Dental College, and by a High-Tech Research Center Project for Private Universities: matching fund Subsidy from MEXT (Ministry of Education, Culture, Sports, Science and Technology) of Japan, 2006–2010. The authors would like to thank Associate Professor Jeremy Williams, Tokyo Dental College, for his assistance with the English of the manuscript.

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