Inhibitory Effect of Antimicrobial Agents on Alginate Production in *Pseudomonas aeruginosa*

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The biofilm formed in local lesions by *Pseudomonas aeruginosa* is mainly composed of alginate and considered to be a barrier against host defense mechanisms and chemotherapeutic agents, causing serious and chronic infections¹⁻³. Alginate is a polymer consisting of β-D-mannurionate and its epimer α-L-guluronate, and *P. aeruginosa* β-D-mannurionate has been characterized to be O-acetylated at a higher rate in comparison with others⁴⁻⁵. Guanosine diphosphomannose dehydrogenase (GMD) is characterized as the key enzyme in alginate formation in *P. aeruginosa*⁶⁻⁸. Therefore, substances that specifically inhibit GMD activity are expected to be effective therapeutic agents against biofilm-forming *P. aeruginosa*. We reported that morphological alteration of mucoid to non-mucoid in *P. aeruginosa* and inhibition on GMD activity might be an useful method to screen alginate biosynthetic inhibitors from microbial metabolites⁹. In this paper we describe mucoid to non-mucoid alteration, inhibition of alginate production, and GMD inhibition by clinically used antibacterial agents and their relationship.

The *P. aeruginosa* PAM38 which constitutively formed mucoid colonies was used for the determination of the minimum concentration inducing non-mucoid alteration (MCINA) and for measurement of alginate production. Minimum inhibitory concentration (MIC) and MCINA were examined by an agar dilution method. Alginate production was estimated by HPLC according to the method of Toyoda et al.¹⁰ and inhibitory rate of alginate formation was expressed by the percent ratio of HPLC peak area. The enzyme GMD was purified from a culture of *P. aeruginosa* strain PAM17 which produced the enzyme in large amounts by the method of Roychoudhury¹¹ with some modifications. The following antibacterial agents were used: erythromycin A (EM-A), clarithromycin (CAM), azithromycin (AZM), oleandomycin (OL), josamycin (JM), gentamicin (GM), clindamycin (CLDM) and chloramphenicol (CP). Penicilllic acid (PCA) was found in the screening system using morphological alteration of mucoid to non-mucoid in *P. aeruginosa*⁹.

In order to know whether the decrease of alginate production resulted from growth inhibition by the antibacterial agents used, the amounts of alginate produced under various concentrations (1/4, 1/16, 1/64 and 1/256 MIC) of the agents were estimated by HPLC analysis. Viable cell counts in all the cultures were not affected under the condition of 1/4 MIC or less of the agents, indicating that a decrease in alginate formation is not concerned by bacterial viability. Table 1 summarizes MIC values of antibacterial agents against *P. aeruginosa*, their inhibitory effects on alginate production, MCINA and GMD activity. As shown in Table 1, low concentration of AZM (0.78 μg/ml), CP (0.78 μg/ml) and PCA (6.25 μg/ml), which corresponds to 1/256 MIC showed strong inhibition of alginate formation, 75, 94 and 85%, respectively. EM-A, CAM, OL and CLDM showed the inhibition dose-dependently. On the other hand, the inhibitory activity by JM and GM was lower level.

As it was predicted that a decrease in alginate formation might result from inhibition of GMD activity, we examined the relation between MCINA activity and inhibitory effect on GMD activity by antibacterial agents. Table 1 summarizes MCINA for PAM38 strain and 50% inhibitory concentrations (IC₅₀) for GMD activity by agents. As expected, inducibility of non-mucoid alteration by each agent except for JM related to the inhibition of alginate formation. The non-mucoid alteration of GM could not be determined because of its antibacterial activity against *P. aeruginosa*. AZM and CP showed a low MCINA (0.78 μg/ml). Inhibitory activities on mucoid formation of EM-A and CAM were relatively low with MCINA 6.25 μg/ml, but that of OL, JM and CLDM were weak (MCINA 100 μg/ml). On the contrary, the IC₅₀ for GMD activity were 1.1 mM for EM-A and 0.6 mM for AZM in which their inhibitory activities...
Table 1. The effects on MIC, inhibition of alginate production, MCINA, and inhibitory activity for GMD of *P. aeruginosa* by antimicrobial agents and penicillic acid.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>MIC (µg/ml)</th>
<th>Inhibition of alginate production (% of control)</th>
<th>MCINA (µg/ml)</th>
<th>50% Inhibition of GMD (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1/4 MIC</td>
<td>1/16 MIC</td>
<td>1/64 MIC</td>
<td>1/256 MIC</td>
</tr>
<tr>
<td>EM-A</td>
<td>200</td>
<td>90</td>
<td>87</td>
<td>48</td>
</tr>
<tr>
<td>CAM</td>
<td>200</td>
<td>72</td>
<td>67</td>
<td>58</td>
</tr>
<tr>
<td>AZM</td>
<td>200</td>
<td>86</td>
<td>76</td>
<td>73</td>
</tr>
<tr>
<td>OL</td>
<td>&gt;1600</td>
<td>94</td>
<td>83</td>
<td>53</td>
</tr>
<tr>
<td>JM</td>
<td>800</td>
<td>45</td>
<td>52</td>
<td>32</td>
</tr>
<tr>
<td>CLDM</td>
<td>&gt;1600</td>
<td>92</td>
<td>89</td>
<td>70</td>
</tr>
<tr>
<td>GM</td>
<td>6.25</td>
<td>17</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>CP</td>
<td>100</td>
<td>93</td>
<td>96</td>
<td>97</td>
</tr>
<tr>
<td>PCA</td>
<td>&gt;1600</td>
<td>91</td>
<td>89</td>
<td>89</td>
</tr>
</tbody>
</table>

*Inhibitory effect on alginate production of OL, CLDM and PCA was examined as MIC: 1600 µg/ml.*

...were not strong. The other agents, OL, JM, CAM, CP, CLDM and GM had almost no GMD inhibitory activity. The inhibitory concentrations above 1.0 mM of CAM and JM could not be determined because of their low solubility in the solvent. No detectable inhibitory activity of CP and PCA on GMD were observed, although the agent strongly inhibited alginate production. The inhibition on alginate production and non-mucoid alteration did not always correlate to the inhibitory activity on GMD, indicating that the inhibition on alginate production of CP and PCA might result from such mode of actions as inhibition of protein synthesis and cytotoxicity, respectively. Kobayashi et al. have reported that the inhibitory effect of EM-A and AZM on GMD is related to a decrease on alginate production. Our results suggest that none of EM, CAM, AZM and CP would be a specific inhibitor of GMD, although these agents resulted in the suppression of alginate production. Tateda et al. have reported that sub-MICs of AZM affect viability and protein synthesis in several strains of *P. aeruginosa*, due to the excellent permeability through the bacterial membrane. Although the possibility remains to be examined, it can be predicted that the high non-mucoid alteration but a weak inhibition of EM-A and AZM on GMD activity, in comparison with OL and JM seems to be due to the difference of permeability through the bacterial membrane, not a direct inhibition on GMD and may be attributable to the suppression of alginate-biosynthesis-related enzyme systems resulting from inhibition of protein synthesis in *P. aeruginosa*.

Elloumi et al. reported that structural analogues of GDP mannose inhibited GMD activity, suggesting that the inhibitors of alginate biosynthesis should be detectable in certain substances relating to enzymatic reactions. So far, there is no report on an inhibitor on alginate biosynthesis from natural products. In order to screen a specific inhibitor on alginate biosynthesis, it needs to examine the effect on other mode of action, in addition to inhibitory activity on GMD activity and mucoid formation.

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