COMMUNICATIONS TO THE EDITOR

A Search for *Pseudomonas* Alginate Biosynthesis Inhibitors from Microbial Metabolites

Sir:

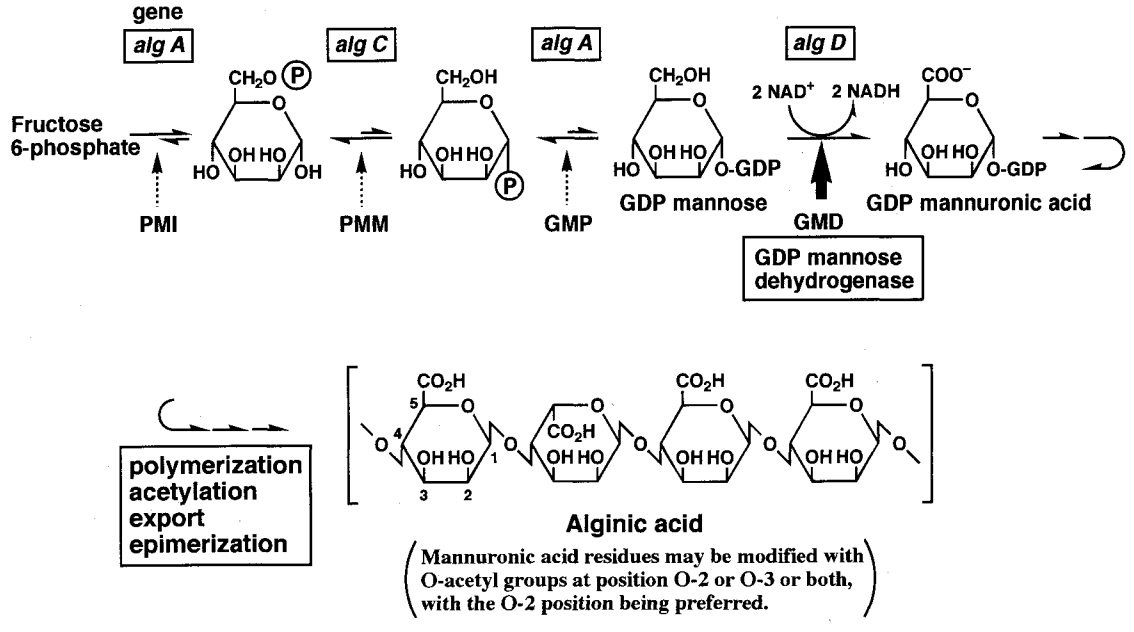
Pulmonary infections caused by *Pseudomonas aeruginosa* (*P. aeruginosa*) often become chronic and incurable especially in diffused panbronchiolitis (DPB) and in cystic fibrosis (CF), when the bacteria form biofilms[1~4]. The biofilm is a complex of host components and bacterial polysaccharides called glycocalyx accumulated on the microbial cell surface. It causes bacterial adhesion to the site of infection and protects organisms from both host-defense mechanisms and therapeutic agents. The bacterial alginate, the main component of the biofilm, is a polysaccharide consisting of the structural units of β-D-mannuronate with *O*-acetyl groups and its C-5 epimer, α-L-guluronate[5~7].

The known enzymes for alginate biosynthesis (Scheme 1) are phosphomannose isomerase (PMI), phosphomannomutase (PMM), guanosine diphosphomannose pyrophosphorylase (GMP), and guanosine diphosphomannose dehydrogenase (GMD)[4,8,9]. As GMD (encoded by the gene *alg* D) is the limiting enzyme for alginate biosynthesis, inhibition of GMD activity would decrease alginate production, resulting in decreased biofilm formation. Consequently, we established a new screening method to detect microbial metabolites that inhibit alginate biosynthesis using mucoid strains of *P. aeruginosa*. The screen is based on the findings that mucoid colony formation is dependent on alginate production and the morphological change of mucoid to non-mucoid colonies occurs concomitantly with a decrease in alginate formation[10]. We were also able to examine the inhibitory effect of microbial metabolites on GMD activity in *P. aeruginosa*, by estimating the amount of alginate produced per bacterial cell using HPLC.

The strain, PAM-38, which produces large amounts of alginate was chosen from clinical isolates of *P. aeruginosa* strains. About 10^7 to 10^8 cfu of PAM-38 from a 5 hour shaking culture in STB broth (Nissui) were seeded on Mueller-Hinton agar plates. Paper disks (8 mm diameter) soaked in test compounds, or the culture filtrates of actinomyces and fungi, were placed on the agar plates where the mucoid strain was seeded. After 18 hours cultivation at 36°C, the morphological change of the bacterial colonies surrounding the disk was examined by eye. Erythromycin A (EM-A), a 14-membered macrolide antibiotic which produced a non-mucoid phenotype in a mucoid strain, was selected as the positive control in the assay. We discovered a microbial metabolite from fungal strain ATF-547 that produced a non-mucoid alteration more distinct than that shown by 25μg/ml of EM-A.

Strain ATF-547 was aerobically cultured in CzS-8 medium (glucose 1.5%, saccharose 1.5%, soybean powder 1.0%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, KCl 0.05%, $FeSO_4 \cdot 7H_2O$ 0.001%, $CaCO_3$ 0.5%, pH 6.0) using a 5-liter mini-jar fermenter for 3 days at 27°C. Isolation and purification of the active component from the culture supernatant were successfully performed by chromatographies on an activated charcoal carbon column and silicagel column, and preparative silicagel

Scheme 1. Biosynthetic pathway of alginic acid in *P. aeruginosa*.



thin layer chromatography. The active compound ($C_8H_{10}O_4$) was identified as penicillic acid (3-methoxy-5-methyl-4-oxo-2,5-hexadienoic acid)[11] from its ^1H and ^{13}C NMR spectral data, as shown in Fig. 1.

Morphological changes on an agar plate (mucoid to non-mucoid alteration) in the mucoid strain PAM-38 are shown in Fig. 2. The minimum concentration of penicillic acid that induced non-mucoid alteration was 6.25 μg/ml. The degree of alteration corresponded to that shown by 6.25 μg/ml of EM-A. In order to know whether the non-mucoid alteration was due to inhibition of alginate formation, the inhibitory activity on GMD, the key enzyme for alginate biosynthesis, was examined. GMD was prepared from *P. aeruginosa* strain PAM-17 which produced a high level of the enzyme. Bacteria were grown with gentle shaking in STB medium using 500-ml Erlenmeyer flasks for 16 hours at 36°C. Purification of GMD from the bacteria collected by centrifugation was carried out following the method of ROYCHOUDHURY *et al.*[12] with slight modifications. Purified GMD was dissolved in 0.5 ml of 100 mM Triethanolamine acetate buffer (pH 7.6), mixed with 0.5 ml of 80% glycerin and kept at −26°C as an enzyme solution. A Tris-HCl buffer solution was prepared by mixing ten μl each of 200 mM dithiothreitol, 100 mM NAD$^+$, 50 mM GDP-D-mannose with 850 μl of 50 mM Tris-HCl buffer at 24°C. Inhibition of GMD was examined according to the procedure of ELLOUMI *et al.*[13] with slight modification. The enzyme reaction was carried out in a mixture of 100 μl of the test sample in 50% methanol, 20 μl of the enzyme solution, and 880 μl of the Tris-HCl buffer for 5 minutes at 25°C. The enzyme activity that consumed 1 μM of NAD$^+$ for 1 minute was determined as 1 unit and the specific activity was expressed as milli units per mg protein. The concentrations that inhibited 50% of GMD activity were 1.2 mM and 2.4 mM, respectively, for EM-A and penicillic acid, correlating with the minimum inhibitory concentration for non-mucoid alterations assayed by the paper disk method. Furthermore, we estimated alginate production by HPLC, in order to examine whether the non-mucoid alteration induced by penicillic acid and EM-A correlates to suppression of alginate formed per cell of *P. aeruginosa*. A culture of the mucoid strain PAM-38 ($10^7 \sim 10^8$ cfu) was overlaid on Mueller-Hinton agar plates containing serial dilutions of either EM-A (μg/ml): 50, 12.5, 3.13, 0.78, or penicillic acid (μg/ml): 400, 100, 25, 6.25, and cultivated overnight at 36°C. Bacteria from the surface of the agar plates were collected in an aliquot of STB broth, and the alginate solution obtained as the supernatant after centrifugation and filtration through

Fig. 1. Structures of EM-A and penicillic acid.

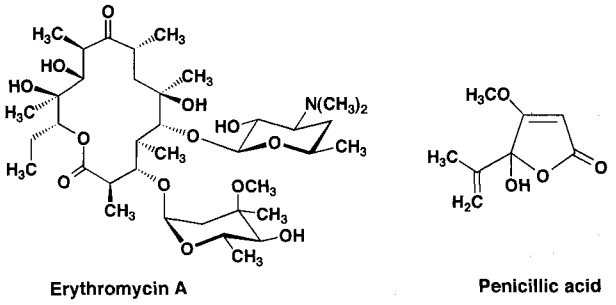


Fig. 2. Morphological change of mucoid to non-mucoid colonies in *P. aeruginosa* strain PAM-38 by EM-A (100 μg/ml) and penicillic acid (100 μg/ml).

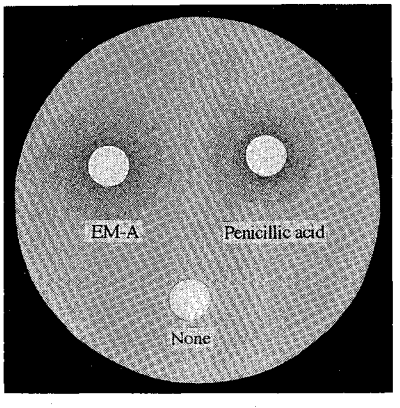
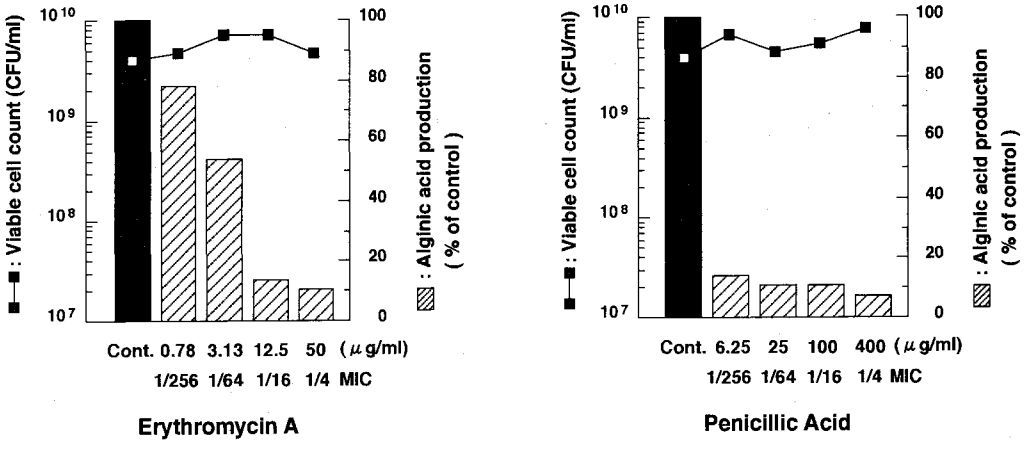


Fig. 3. Inhibitory effects on alginic acid production in *P. aeruginosa* strain PAM-38 by EM-A and penicillic acid.



membrane filters. The alginate solution thus prepared was boiled in $CuSO_4$-HCl solution with naphthoresorcinol reagent and the alginate was extracted by *n*-butyl acetate at room temperature. The extract was concentrated to dryness, to provide a residue containing alginate. The amount of alginate was estimated by HPLC (Senshu Pack C-18 in the solvent system; acetonitrile : *n*-butyl acetate : H_2O = 75 : 5 : 20, UV detection : λ 565 nm)[14]. The quantity per bacterial cell was determined as a ratio of the peak area of alginate shown by HPLC to number of bacterial cells, and compared with that of the control with no antibiotic. Low concentrations of EM-A (12.5 μg/ml) and penicillic acid (6.25 μg/ml) inhibited alginate production in strain PAM-38, by 86% (Fig. 3). These concentrations of EM-A and penicillic acid correspond to 1/16 and 1/256 of the respective MICs. Therefore the alteration of mucoid bacteria to a non-mucoid phenotype and the GMD inhibition were correlated to inhibition of alginate production per bacterial cell. Furthermore, our data suggest that the morphological change associated with the non-mucoid phenotype would be a simple and useful method to search for alginate biosynthesis inhibitors from microbial metabolite.

This is the first report that penicillic acid inhibits alginate formation, although the mechanism is not yet solved. Similar effects of several antibiotics on GMD activity and alginate production in mucoid strains of *P. aeruginosa* are under investigation.

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