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Effect of antibiotics on extracellular protein level in *Pseudomonas aeruginosa*

Eigo Takahashi [a,b], Jae Man Lee [b], Hiroaki Mon [b], Yuuka Chieda [a], Chisa Yasunaga-Aoki [a], Takahiro Kusakabe [b], Kazuhiro Iiyama [a,*]

[a] Laboratory of Insect Pathology and Microbial Control, Institute of Biological Control, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka, Japan
[b] Laboratory of Insect Genome Science, Faculty of Agriculture, Graduate School, Kyushu University, Fukuoka, Japan

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ABSTRACT

Pseudomonas aeruginosa PAO1 organisms harbouring different plasmids were cultured in broths containing appropriate antibiotic(s). Extracellular proteins were more abundant in the presence of tetracycline or kanamycin than in the presence of other antibiotics. Zymography revealed that alkaline protease (AprA) production was interfered by these antibiotics. Extracellular proteins were not observed at the same level when AprA-deficient EG03 strains were cultured in the presence of different antibiotics. The extracellular protein levels were dependent on the antibiotics and plasmid derivative groups. Levels of extracellular protein were not significantly different between PAO1 (pBBR1MCS-5) and EG03 (pAprcomp-MCS5), and profiles of the extracellular proteome were comparable. In contrast, the level of EG03 (pBBR1MCS-MCS5) extracellular protein was higher than those observed in the other two strains. These results suggested that although AprA partially contributes to the alteration of extracellular protein level, the effect is limited.

1. Introduction

Pseudomonas aeruginosa is ubiquitous and found in diverse types of environment including soil, freshwater, and marine environments. It is also a known opportunistic pathogen of vertebrates, invertebrates, and plants (Jander et al., 2000; Jarrell and Kropinski, 1982). *P. aeruginosa* secretes various proteins as virulence factors such as exotoxin A (ToxA), ExoU, ExoS, ExoY, ExoT, and proteases (Gellatly and Hancock, 2013). ToxA is an ADP-ribosyltransferase that inactivates the elongation factor 2 (Iglewski et al., 1977). ExoS and ExoT are bifunctional cytotoxins (Goehring et al., 1999; Yahr et al., 1996). ExoY and ExoU are an adenylate cyclase and a potent phospholipase A_2, respectively (Yahr et al., 1998; Phillips et al., 2003; Sato et al., 2003). Furthermore, *P. aeruginosa* produces various proteases such as elastase A, elastase B, alkaline protease, protease IV, and *P. aeruginosa* small protease (Hoge et al., 2010).

To investigate the contribution of these extracellular proteins in *P. aeruginosa* pathogenesis, the pathogenicity of gene-disrupted and complemented strains is often compared with that of the parent strain. To prevent plasmid curing, an appropriate antibiotic is often added when the plasmid is used for gene complementation. Therefore, misinterpretation of results may occur if the antibiotic causes an alteration in the extracellular protein level.

During the study of *P. aeruginosa* extracellular protein, certain antibiotics were found to cause a dramatic change in the extracellular protein levels. A previous study showed that tetracycline inhibited protease production, but not protease activity, in *P. aeruginosa* (Shibl and Al-Sowaygh, 1980). Furthermore, erythromycin suppressed leucocidin, elastase, and protease production in *P. aeruginosa* (Kita et al., 1991), whereas aminoglycoside antibiotics, including gentamicin and streptomycin, decreased the levels of protease and phospholipase C (Hostacká and Majtán, 1993). Lincomycin and clindamycin inhibited lipase production in *Propionibacterium* spp., whereas tetracycline only inhibited its production in *Propionibacterium granulosum* (Unkles and Gemmell, 1982).

In eukaryotic cells, tetracycline is known to inhibit matrix metalloproteases (Greenwald et al., 1992; Nip et al., 1993; Duivenvoorden et al., 1997; Maitra et al., 2003; Acharya et al., 2004). Therefore, because *P. aeruginosa* alkaline protease (AprA), encoded by *aprA*, is a metalloprotease (Morihara, 1964; Okuda et al., 1990; Duong et al., 1992), antibiotics are thought to inhibit its production. We speculated that secreted AprA may degrade other extracellular proteins in the absence of an antibiotic. Conversely, if AprA production is suppressed by certain antibiotics, degradation of extracellular proteins is either reduced or does not occur. To investigate this hypothesis, extracellular proteins of *P. aeruginosa* cultured in the presence of several antibiotics were assessed in this study.

* Corresponding author at: Institute of Biological Control, Faculty of Agriculture, Graduate School, Kyushu University, Hakozaki 6-10-1, Higashi-ku, Fukuoka, Japan.
E-mail address: iiyama@grt.kyushu-u.ac.jp (K. Iiyama).

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Table 1
Bacteria, plasmids and oligonucleotides used in this study.

Bacterium, plasmid or oligonucleotide	Description or sequence[a]	Reference or source
Escherichia coli		
DH5α	F$^-$, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(r_K^-, m_K^+), *phoA*, *supE*44, λ$^-$, *thi*-1, *gyrA*96, *relA*1	Laboratory stock
S17-1	*thi*, *pro*, *hsdR*, *recA*, chromosomal RP4, *tra*$^+$, Tp^R, Sm/Sp^R	Simon et al. (1983)
Pseudomonas aeruginosa		
PAO1	Prototype strain	Professor J. Kato
PrEG03	*aprA* deletion mutant of PAO1, Δ*aprA*::(FRT-Ω*aacC4*-FRT), Gm^R	This study
EG03	Markerless *aprA* deletion mutant of PAO1, Δ*aprA*::FRT	This study
Plasmid		
pBBR1MCS-2	Broad host range plasmid, Km^R	Kovach et al. (1995)
pBBR1MCS-3	Broad host range plasmid, Tc^R	Kovach et al. (1995)
pBBR1MCS-4	Broad host range plasmid, Amp/Cb^R	Kovach et al. (1995)
pBBR1MCS-5	Broad host range plasmid, Gm^R	Kovach et al. (1995)
pJB3	Broad-host-range cloning vector, Amp/Cb^R	Blatny et al. (1997)
pJB3Km1	Broad-host-range cloning vector, Km^R, Amp/Cb^R	Blatny et al. (1997)
pJB3Tc20	Broad-host-range cloning vector, Tc^R, Amp/Cb^R	Blatny et al. (1997)
pHERD20T	*Escherichia-Pseudomonas* shuttle vector, Amp/Cb^R	Qiu et al. (2008)
pHERD26T	*Escherichia-Pseudomonas* shuttle vector, Tc^R	Qiu et al. (2008)
pHERD30T	*Escherichia-Pseudomonas* shuttle vector, Gm^R	Qiu et al. (2008)
pK18*mobsacB*	Allelic-exchange suicide vector, *sacB*, *oriT* (RP4), *lacZ*, Km^R	Schäfer et al. (1994)
pAprC	9.1-kb *aprX-aprD-aprE-aprF-aprA* fragment in pK18*mobsacB*, Km^R	This study
pKOC	10.2-kb *aprX-aprD-aprE-aprF-ΔaprA*::(FRT-*aacC4*-FRT) fragment in pK18*mobsacB*, Km^R	This study
pAprcomp-MCS5	9.1-kb *aprX-aprD-aprE-aprF-aprA* fragment in pBBR1MCS-5, Gm^R	This study
pHP45Ω	Source of Ω, Amp^R, Sm/Sp^R	Prentki et al. (1991)
pHP45Ω*aacC4*	Source of Ω*aacC4*, Amp^R, Gm^R	Blondelet-Rouault et al. (1997)
pPS854	Source of FRT sequence, Amp^R	Hoang et al. (1998)
pPS854aacC4	Source of FRT-*aacC4*-FRT cassette, Ω*aacC4* fragment in pPS854, Amp^R, Gm^R	This study
pGEM T-Easy	TA cloning vector, Amp^R	Promega
pGEMFRTaacC4	FRT-*aacC4*-FRT cassette in pGEM T-Easy, Amp^R, Gm^R	This study
pFLP2	Broad-host-range, site-specific excision vector, *ori1600*, *oriT*, *sacB*, Amp^R	Hoang et al. (1998)
pFLP2ΩSm/Sp	Ω fragment in pFLP2, Amp^R, Sm/Sp^R	This study
Oligonucleotide		
K18MSLEf	GAATTCCATGTCATAGCTGTTTCCTGTG	This study
K18MSLEr	GAATTCCACTGGCCGTCGTTTTACA	This study
PA1244(53R)-IF	TATGACATGGAATTCGTCTTTTCCTTTTCATCCTTCGTCA	This study
aprF1000f	ATGGAGAAGAGCCATTACGACCT	This study
aprI(86R)-IF	CGGCCAGTGGAATTCATCAGACTGCTGGCCATACTGATAC	This study
omega-inner	TATGCTTGTAAACCGTTTTGTGAA	This study
FRT-EcoRV	GATATCAAGCTTGCATGCCTGCAGGTCGACTCT	This study

[a] Abbreviations for phenotype: Amp^R, ampicillin resistance; Cb^R carbenicillin resistance; Gm^R gentamicin resistance; Km^R kanamycin resistance; Sp^R, spectinomycin resistance; Sm^R, streptomycin resistance; Tc^R, tetracycline resistance; and Tp^R, trimethoprim resistance. Underlines in oligonucleotides indicated artificial sequences of restriction enzyme recognition site or overlapping vector sequences for In-fusion.

2. Materials and methods

2.1. Bacteria, plasmids, and culture condition

Bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 was a gift from Professor J. Kato (Hiroshima University, Japan). The strain was originally obtained from the laboratory of Ananda M. Chakrabarty (University of Illinois at Chicago, USA). Cultures of *Escherichia coli* and *P. aeruginosa* were routinely grown in Luria–Bertani (LB) medium (Lennox; Sigma-Aldrich, Japan) at 37 °C and 30 °C, respectively. For protein analysis, *P. aeruginosa* was inoculated into 3 ml LB broth in a glass test tube (inner diameter 13 mm × length 125 mm). The tube was aerobically incubated (FMS-100; Tokyo Rikakikai Co., Ltd., Japan) with a reciprocal shaker (stroke width of 25 mm; Multi Shaker MMS-310; Tokyo Rikakikai Co., Ltd., Japan) at 200 strokes/min for 48 h. Antibiotics were used at the following concentrations (for *E. coli* and *P. aeruginosa*, respectively): 30 and 50 μg/ml for gentamicin, 12 and 48 μg/ml for tetracycline, and 30 and 200 μg/ml for kanamycin. For β-lactam antibiotics, ampicillin (50 μg/ml for *E. coli*) and carbenicillin (200 μg/ml for *P. aeruginosa*) were used. Triclosan was added to the medium at 5 μg/ml for *P. aeruginosa* selection. Streptomycin was used for *E. coli* S17-1 culture at 5 μg/ml.

Plasmids for *aprA* disruption and complementation were constructed as follows: Ω*aacC4* fragment, amplified by PCR using a primer (omega-inner) from pHP45Ω*aacC4*, was ligated into *Eco*RV-digested pPS854, and designated as pPS854aacC4. To introduce *Eco*RV sites into both ends of the FRT-*aacC4*-FRT cassette in pPS854aacC4, PCR was carried out using the FRT-EcoRV primer and the KOD-Plus-Neo polymerase (TOYOBO Co., Japan). After adding adenine overhangs to the amplicon using a HybriPol DNA polymerase (Nippon Genetics Co. Ltd., Japan), the fragment was cloned into pGEM T-Easy (Promega KK, Japan) to construct the pGEMFRTaacC4.

pK18*mobsacB* was linearised by PCR with K18MSLEf/K18MSLEr primers using the KOD-Plus-Neo polymerase. The 9.1-kb *aprX–aprD–aprE–aprF–aprA* fragment was amplified from *P. aeruginosa* PAO1 genomic DNA using PA1244(53R)-IF/aprI(86R)-IF primers. Purified PCR fragment was cloned into the linearised pK18*mobsacB* using the In-fusion PCR cloning kit (Takara Bio Inc., Japan) to create pAprC. FRT-Ω*aacC4*-FRT cassette was prepared from pGEMFRTaacC4 by *Eco*RV digestion. The cassette was ligated into *Eco*RV-digested pAprC to create the pKOC (*aprA*-disruption plasmid).

The 9.1-kb *aprX–aprD–aprE–aprF–aprA* PCR fragment was cloned into linearised pBBR1MCS-5 using a similar procedure. The plasmid was designated as pAprcomp-MCS5 (*aprA*-complementation plasmid).

pHP45Ω was digested with *Bam*HI, and Ω fragment carrying the Sm/Sp resistance gene was ligated into the same site on pFLP2 to create the pFLP2Sm/Sp. Flp recombinase expressed from pFLP2Sm/Sp excised FRT-Ω*aacC4*-FRT cassette by site-specific recombination (Hoang et al., 1998).