ISSN 0026-2617, Microbiology, 2011, Vol. 80, No. 6, pp. 768–774. © Pleiades Publishing, Ltd., 2011.

EXPERIMENTAL ARTICLES

A Natural Antisense Transcript Regulates *muc*D Gene Expression and Biofilm Biosynthesis in *Pseudomonas aeruginosa*[1]

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Received July 27, 2010

Abstract—Natural antisense transcripts (NATs) are naturally widespread and have very important functions in regulating expression of their target genes. In this report, we identified a novel NAT, designated *muc*D-AS, transcribed from the opposite strand of *muc*D gene locus, which has a pivotal function in regulating the biosynthesis of alginate. For the biotechnological and medical significance in the study of alginate biosynthesis, we investigated the effect of *muc*D-AS on *muc*D expression and alginate biosynthesis, and confirmed *muc*D-AS can induce biofilm formation of *P. aeruginosa* significantly. Since alginate has a key function in the infection process and acts as virulence factor of *P. aeruginosa*, we speculate *muc*D-AS may have a regulatory function in this process.

Key words: Natural antisense transcripts; *muc*D-AS; alginate; biofilm; *Pseudomonas aeruginosa*

DOI: 10.1134/S0026261711060245

Natural antisense transcripts (NATs) are endogenous antisense transcripts occurring naturally, defined as any RNA transcript that is complementary to another endogenous transcript [11]. When both strands of DNA are transcribed from a single locus, there are complementary regions between the sense RNA and the NATs. NATs are capable of regulating prokaryotic and eukaryotic gene expression and exert their regulatory effects at multiple levels, including transcription, RNA editing, post-transcription, and translation. The wide distribution and conservation of NATs strongly suggest that NATs are not accidental and may play a general regulatory role in gene expression in prokaryotes and eukaryotes [2, 3].

P. aeruginosa is an important opportunistic pathogen causing serious infections in immunocompromised patients. Eucaryotic tissue damage caused by *P. aeruginosa* is due to the production of several extracellular virulence factors and cell-associated virulence factors by the bacteria. The ability to form biofilms is the crucial factor in fatal infections by *P. aeruginosa* and has made this bacterium a model organism with respect to biofilm formation [4–7].

*Muc*D located in the *muc*ABCD gene cluster of *P. aeruginosa* encodes a serine proteinase involved in transcriptional regulation of alginate biosynthesis related genes. Among them, *muc*D acts as a negative regulator of alginate production in *P. aeruginosa* and its mutant snowed enhanced induction of *alg*D transcription, which encodes a key enzyme of alginate biosynthesis. Alginate is a secretory exopolysaccharide, consisting of variable amounts of 1,4-linked-D-mannuronate and its α-L-guluronate, functions as extracellular matrix in biofilm formation and plays an important role during infection by *P. aeruginosa* in animals [8, 9].

In our previous work, we screened a previously described NAT, complementary to *muc*D mRNA [15], speculating that it may act as a potential negative regulator of *muc*D mRNA and a regulator in alginate biosynthesis and biofilm formation of *P. aeruginosa*. The present study confirms our hypothesis.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. *P. aeruginosa*, PAO1. Type strain ATCC 6872; its derivative, missing *muc*D gene; and a *muc*D partial deletion mutant which retained *muc*D-AS corresponding DNA region was assayed for its ability to initiate alginate biosynthesis and biofilm formation. All strains, along with their relevant characteristics and sources, are listed in Table 1. The wild-type strains and mutants were cultured on LB medium (containing 10 g of bacto-tryptone, 5 g of the yeats extract and 10 g NaCl per 1) at 37°C. To screen transformed *E. coli*, for pGEM-T vector, 50 μg/ml of ampicillin was added to the LB medium.

Sense and antisense probes RNA Preparations, Northern Blotting. Digoxin-labeled sense and antisense single stranded complementary RNA probes of *muc*D-AS and *muc*D mRNA were generated by in

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
P. aeruginosa		
PAO1	Wild type, non-mucoid, Carb^s	Collection
PAMA	PAO1 _*muc*D-AS::bla, mucoid, Carb^r	This study
PAMD	PAO1 _*muc*D:bla, mucoid, Carb^r	Collection
Plasmids		
pGEM-mAS	pGEM clone vector containing mucD-AS fragment	Previous study
pJQ200SK	a rescued plasmid, Amp^r	Collection
pUCP	*E. col—P. aeruginosa* shuttle vector, Carb^r	Collection
pUMA	*E. coli—P. aeruginosa* shuttle vector, Spr 24	This study

vitro transcription of linearized plasmid. The DNA template was the fragment containing the sequence of *muc*D-AS excised from pGEM-mAS plasmid by *Sal*I/*Nco*I hydrolysis. The antisense probe was generated by SP6 transcription of a *Sal*I linearized template, and the sense probe by T7 transcription of a *Nco*I linearized template. The transcription and purification of sense and antisense probe RNA were carried out using DIG RNA Labeling Kit (Roche Diagnostics) according to the manufacturer's manual. Total RNA was extracted using Trizol reagent (Invitrogen) from PAO1 cell culture. RNA was purified using the RNeasy Mini Kit (QIAGEN). 12 micrograms of total RNA was separated on a 1% formaldehyde-MOPS agarose gel. The samples were electroblotted onto nylon membrane (Hybond-N$^+$, Roche), and cross-linked under ultraviolet radiation. The membranes were pre-hybridized for 4 h at 65°C in standard hybridization buffer with formamide (50% formamide, 5× sodium chloride/sodium citrate buffer (SSC), 0.1% lauroylsarcosine, 2% blocking reagent, 0.02% sodium dodecylsulfate (SDS), 100 μg/ml sheared salmon sperm DNA). Hybridization was performed at the same condition for 18 h after addition of above digoxin (DIG)-labeled probes, After stringent washes at room temperature for 30 min in 0.1% SDS, 2 × SSC, and at 68°C for 30 min in 0.1% SDS and 0.1 × SSC, the corresponding target RNA was detected by chemiluminescence using CSPD ready-to-use reagent (Roche) and autography according to the user's manual.

Construction of *muc*D partial deletion mutant by replacement with the carbenicillin resistance cassette. To determine if the endogenous mucD-AS affected biofilm formation, we constructed a mutant with a partial deletion of *muc*D locus from wild-type strain PAO1 using an insertion-deletion strategy, the region from 605 bp to 756 bp of *muc*D ORF was replaced by carbenicillin cassette (bla). This mutant has an inactivated *muc*D gene but retains *muc*D-AS sequence. The primers used to construct and confirm gene deletion are listed in Table 2. Briefly, a 1.425 bp fragment containing *muc*D ORF was amplified from PAO1 genomic DNA by using primers *muc*D-P1 (containing a *Not*I site at its 5' end) and *muc*D-P2 (containing an *Apa*I site at its 5' end). A carbenicillin resistance marker fragment (*bla*), containing 981 bp from pUC18, was amplified using primers *bla*-P3 (with a *Cla*I site at the 5' end) and *bla*-P4 (with a *Bg l*II site at the 5' end). These amplicons were subjected to *Cla*I/*Bgl*II digestion and subsequent ligation to insert *bla* cassette into the internal region from 605 bp to 756 bp of *muc*D which also subjected to *Cla*I/*Bgl*II digestion and produce a *muc*D-left::bla::*muc*D-right fragment. Then this fragment and a plasmid pJQ200SK were digested by *Not*I and *Apa*I enzymes and ligated to produce a recombinant suicide vector pJQ-MBla. The ligated product was used directly for the transformation of PAO1. Template genomic DNA was prepared from transformants selected on agar plates containing 5% sucrose and 50 μg/ml carbenicillin. Confirmation of the bla presence at the desired locus was performed by PCR and Southern blot. Primers *muc*D-P1 and *muc*D-P2 were used to amplify fragments with the correctly predicted sizes from the mutants. Wild-type PAO1 chromosomal DNA was used as a negative control.

Southern blot analysis. Chromosomal DNA was extracted using the genomic DNA purification kit (Promega) following instructions from the manufacturer and digested with *Hind*III, the digested products were subjected to electrophoresis for 1 h. DNA transfer, digoxigenin labeling of the probe, and chemiluminescence detection were carried out according to the instructions provided by the manufacturer (Roche). Digoxin-labeled PCR product of *muc*D and *bla*, generated with primers *muc*D-P1/*muc*D-P2, *bla*-P3/*bla*-P4, were used as the probe in Southern blot analysis.

Table 2. Primers used to construct the partial *muc*D deletion mutant by PCR restriction-ligation mutagenesis

Primer	Nucleotide sequence (5' ⟶ 3')	Amplicon (bp)
*muc*D-P1	5'AG*GCGGCCGC*ATGCATACCCTAAAACGCTGTAT 3'	1425
*muc*D-P2	5'AG*GGGCCC*TTATTCGGCCAGC TTGAAGGTAAT 3'	
bla-P3	5'GCG*ATCGAT*TCAGTGAGGCACCTATCTCAGCGA 3'	981
bla-P4	5'GCG*AGATCT*AAATGTGCGCGGAACCCCTAT 3'	

The sequences indicated in italic and underline are restriction enzyme sites.

Biofilm observation by CSLM. First, biofilm formation was confirmed by electron microscopy to distinguish biofilm producers from non-producers. For this operation, the transformed bacteria were grown in six well plates on glass coverslips at 35°C in 5% CO_2 for 48 h with LB medium then they were fixed in Trumph's fixative overnight [10]. After fixation, these samples were dehydrated in ascending series of ethanol, followed by air drying and mounted on metallic stubs. Then 1 ml concanavalin A (ConA) from *Canavalia ensiformis* labelled with fluorescein isothiocyanate (FITC-ConA, Sigma) solution was introduced into the cell (ConA has an affinity for glucose and mannose residues). After being stained for 30 min in dark and then washed twice with PBS to remove excess stain, the samples were incubated for 15 min with Propidium Iodide (PI, Jinmei Biotech) in the same condition as above to label the nucleoid region of the cells. The biofilm samples were then viewed under the confocal scanning laser microscopy [11] (CSLM, Leica).

Alginate production assays. Because the cell number of different sample varies greatly, we used the alginate/total protein ratio of each sample as alginate production value. The extraction of exopolysaccharide (alginate) was performed as described by Pedersen et al [12]. Sample of the cultures (1 ml) were centrifuged at 20000 *g* for 30 min at 4°C. The supernatant was heated for 30 min at 80°C and was centrifuged at 20000 *g* for 5 min at 4°C. The pellet was discarded, and the supernatant containing the alginate was precipitated with ice-cold 99% ethanol (3 × volume). After 1 to 2 h at 4°C, the precipitated alginate was collected and dissolved in 1 ml sterile 0.9% saline. The content of uronic acid polymers (the component of alginate) in the samples was then analyzed by the carbazole-borate assay with D-mannuronlactone (Sigma) as a standard. Briefly, 118 μl of the sample was mixed with 1 ml of borate-sulfuric acid reagent (100 mM H_3BO_3 in concentrated H_2SO_4) on ice, and 34 μl of carbazole reagent (0.1% in ethanol) was added. The mixture was heated to 55°C for 30 min, and the absorbance (OD_{530}) was measured. Bradford assay was used for the protein determination of each sample with bovine serum albumin (BSA) as the standard, the alginate/protein ratio of each sample was considered as the value of alginate production [13].

Statistical analysis. Results in the figures are representative of at least three independent experiments yielding similar findings and are expressed as mean ± standard deviation. Differences were analyzed by Student *t* test. Comparisons were made using a significance level of $P < 0.01$.

RESULTS

Northern blot analysis. In our previous work [15], we isolated a 269nt NAT (designated *muc*D-AS) complementary to *muc*D mRNA (Fig. 1). To confirm the existence of *muc*D-AS, Northern blotting was carried out with total RNA extracted from PAO1, using a DIG-labelled probe specific for *muc*D and *muc*D-AS transcripts respectively, the corresponding bands of *muc*D mRNA and *muc*D-AS were detected (Fig. 2), these resutlts validate the existence of *muc*D-AS.

Construction of mutant PAO1 mutant strain. To test whether *muc*D-AS plays a role in biofilm development in PAO1, we utilized standard genetic strategies to disrupt *muc*D but retain *muc*D-AS in PAO1. A recombinant suicide vector pJQ-MBla containing a *muc*D-left::bla::*muc*D-right fragment was constructed after a serials of cloning, which was then transformed into PAO1. The transformants were grown at 30°C with antibiotic and sucrose selection, and 7 resistant clones were isolated, but only 1 integration of pJQ-MBla into

835

mucD ORF sequence GTGAACAAGGATCTCGCCGAGTCCTTCGGCCTCGACAAGCCGTCCGGC

mucD-AS*c a c t t g t t c c t a g a g c g g c t c a g g a a g c c g g a g c t g t t c g g c a g g c c g*

GCGCTGGTGGCGCAACTGGTGGAAGACGGCCCGGCGGCCAAGGGTGGCCTGCAGGTGGGCGATGTGATCCTCAGCCTGAA

c g c g a c c a c c g c g t t g a c c a c c t t c t g c c g g g c c g c c g g t t c c c a c c g g a c g t c c a c c c g c t a c a c t a g g a g t c g g a c t

t

CGGCCAGTCGATCAACGAGTCCGCCGACCTGCCGCACCTGGTGGGCAACATGAAGCCGGGCGACAAGATCAACCTGGACG

g c c g g t c a g c t a g t t g c t c a g g c g g c t g g a c g g c g t g g a c c a c c c g t t g t a c t t c g g c c c g c t g t t c t a g t t g g a c c t g

c

1103

TGATTCGCAACGGCCAGCGCAAGTCCCTGAGCATGGCGGTAGGCAGCCTTCCGGACGACGA

a c t a a g c g t t g c c g g t c g c g t t c a g g g a c t c g t a c c g c c a t c c g t c g g a a g g c c t g c t g c t

Fig. 1. *Muc*D and *muc*D-AS sequences. The arrows indicate the start/termination sites and direction of *muc*D-AS transcription. Uppercase letters represent partial cDNAsequence of *muc*D sequences shown in a 5' to 3' sense orientation, whereas lowercase boldface letters represent the *muc*D-AS sequences presented in a 3' to 5' direction. The antisense is located between nucleotide 835 and 1103 of *muc*D ORF sequence. Such that the complementary antisense relationship between *muc*D and *muc*D-AS is revealed.

the *muc*D gene was confirmed by PCR and Southern blot (Fig. 3). Analysis of the resulting amplicons of this mutant revealed that pJQ-MBla had been inserted in the genome via a double crossover event and that expression of the *bla* fragment had occurred. As expected, integration resulted in the loss of *muc*D expression.

Biofilm observation by CSLM and alginate production assays. Biofilm formation for 48 h by wild-type (PAO1), *muc*D-AS mutant (PAMA) and *muc*D mutant (PAMD) are significantly different (Fig. 4, Fig. 5), which suggests a strong correlation between *muc*D-AS activity and alginate production. The PAO1 wild-type is nonmucoid and has no visible biofilm formation (Fig. 4-1).While transfected with pUMA, which means the expression of *muc*D-AS was upregulated, the transformants obviously have more alginate formation than that of wild-type (Fig. 4-2), and the same phenotypes were observed in the *muc*D full length or partial knockout mutant (Fig. 4-2, 4-3 and 4-4), the alginate production assay also got a coincident result (Fig. 5). Furthermore, this obviously different phenotype was not due to differences in the growth rate of PAO1 and transfectant or mutant, as they had similar growth rates (data not shown), so we speculated the expression of *muc*D-AS may upregulate the expression of one or more genes which are necessary for the biofilm biosynthesis.

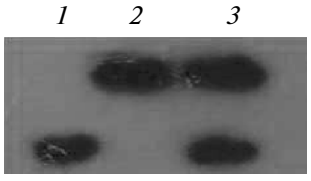

Fig. 2. Northern blot analysis of PAO 1 *muc*D-AS and *muc*D mRNA. Total RNA (10 μg) from PAO1 were separated on a 1.2% formaldehyde agarose gel, transfered onto a nylon membrane, then hybridized to a DIG-labelled probespecific for *muc*D-AS (*1*), *muc*D mRNA (*2*), and the mixed probes for *muc*D-AS and *muc*D mRNA (*3*) transcripts.

DISCUSSION

Over the past few years, antisense ranscription in eukaryotic genomes has become increasingly evident due to the availability of the high throughput sequencing technologies and bioinformatics tools [2, 3, 14–17]. In our previous study, we got some novel NATs molecules based on RNase I protection assay. Among them we focused on an interesting NAT, designated as *muc*D-AS, which is complementary to *muc*D mRNA and confirmed that *muc*D-AS plays an important role in the regulating of alginate biosynthesis of PAO1. Because alginate has a key function in the infection process and acts as virulence factor of *P. aeruginosa*, we speculate that *muc*D-AS may have a regulatory function in this process.

We only investigated the results for *muc*D expression and alginate biosynthesis expression when the expression of *muc*D-AS was increased or decreased, but its accurate mechanism are still unkown. So in the future further analyses will be necessary to completely understand the mechanisms of this action.

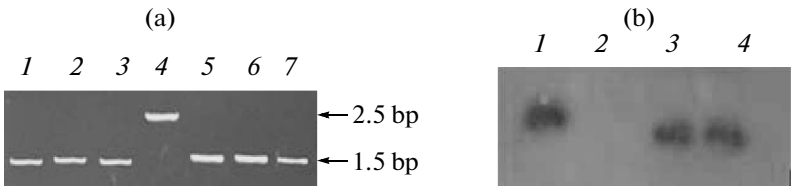

Fig. 3. The screening of *muc*D mutant. (a) – PCR amplification of *muc*D gene from *1–7* clone genomic DNA, and lane *4* indicates clone 4 has a 2.500 bp recombinant *muc*D gene; (b) – Southern blot of mutant. Lane *1–2* corresponding to *muc*D/bla specific probe hybridized with genomic DNA of PAO1; lane *3–4* is the same probe hybridized with genomic DNA of mutant.

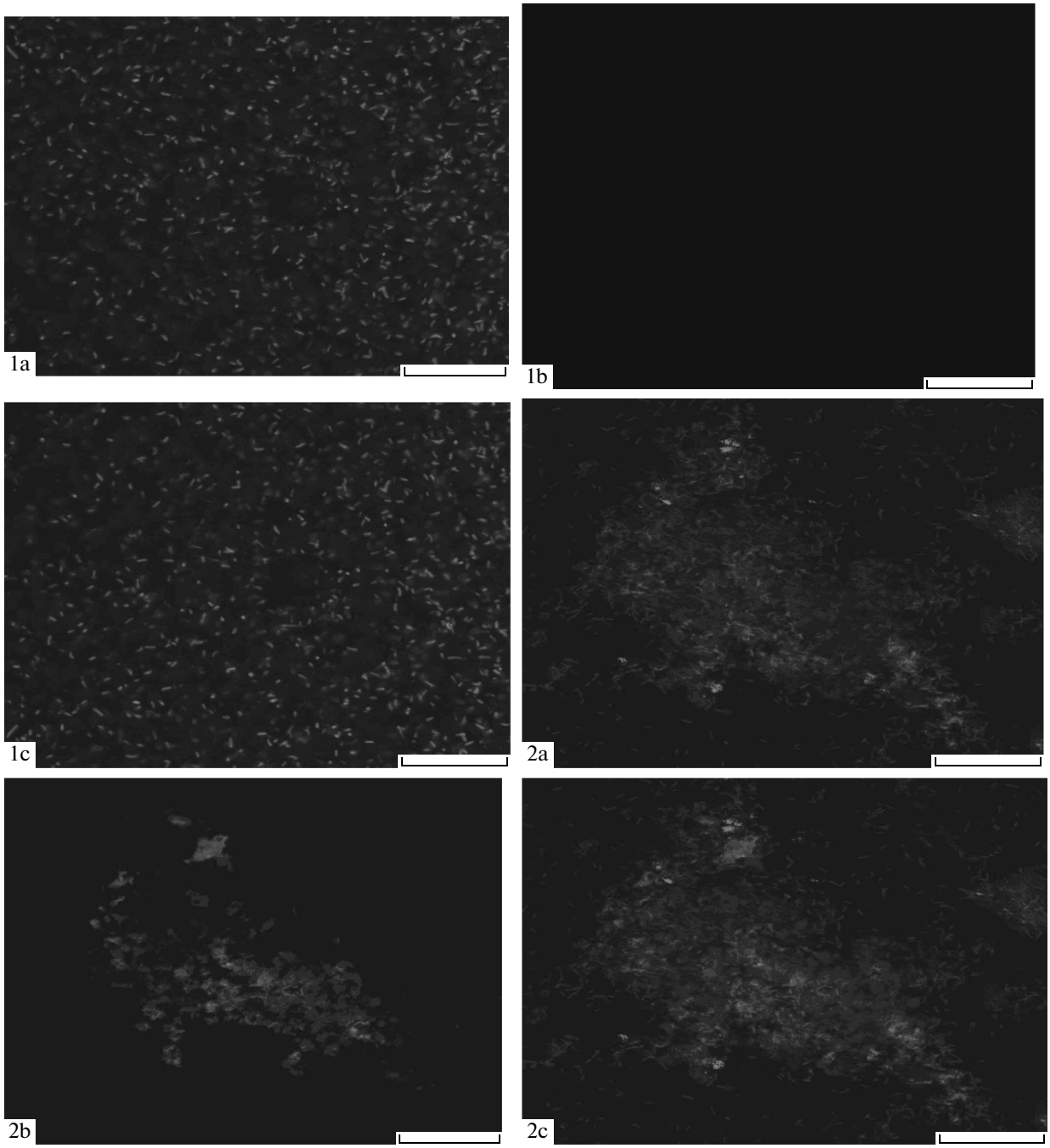

Fig. 4-2. PI/FITC-Con A double staining results of PAO1 transfected with pUMA. 2a – PI staining; 2b – FITC-Con A staining; 2c – in the merged images, nuclei staining is white, alginate staining is gray. The follwing 3c and 4c is in the same pattern.

Fig. 4-3. Staining results of *muc*D-AS mutation strain PAMA. 3a – PI staining; 3b – FITC-Con A staining; 3c – PI/FITC-Con A double staining.

Fig. 4-4. Staining results of *muc*D mutation strain PAMD. 4a – PI staining; 4b – FITC-Con A staining; 4c – PI/ FITC-Con A double staining.

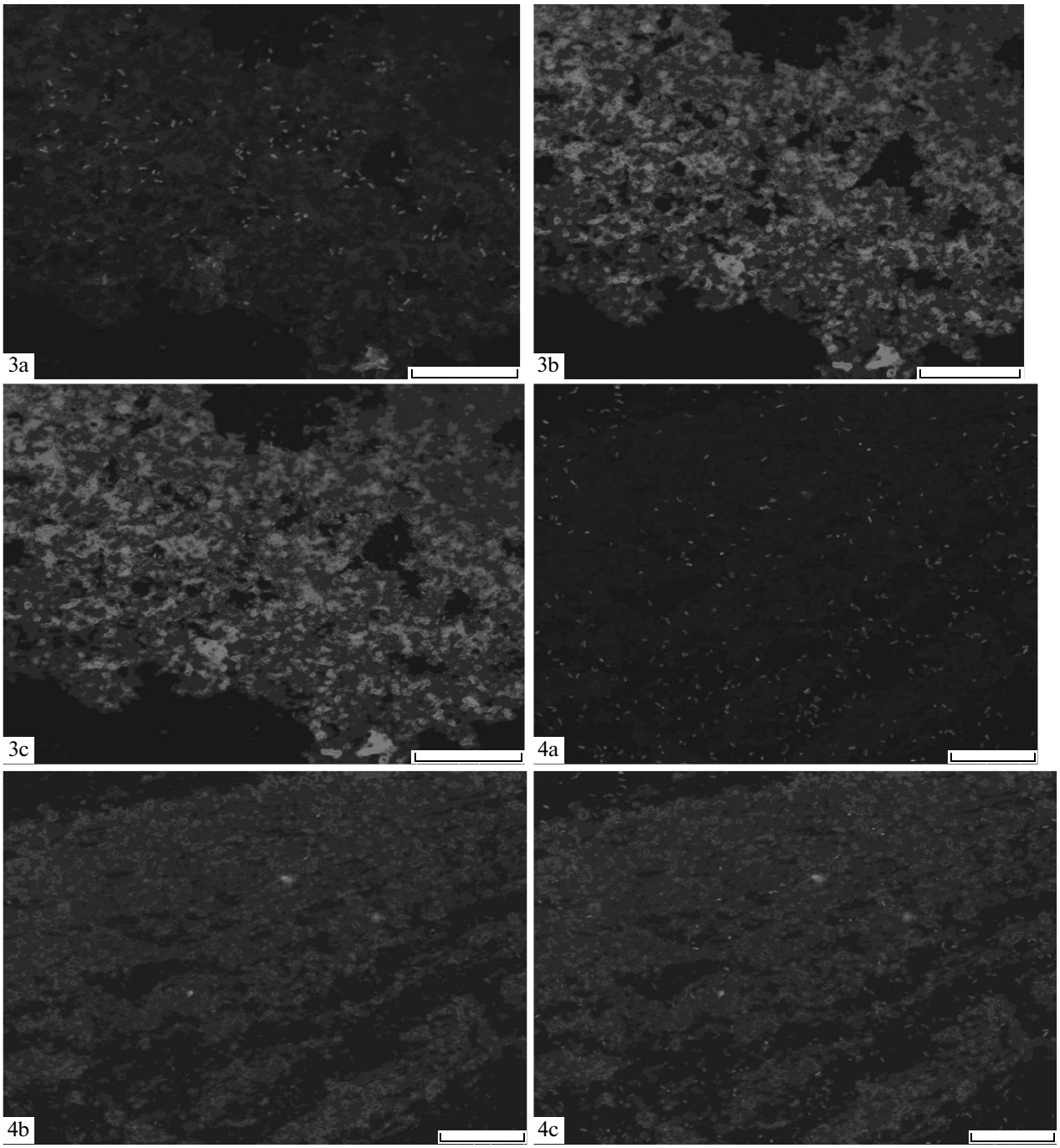

Fig. 4. Contd.

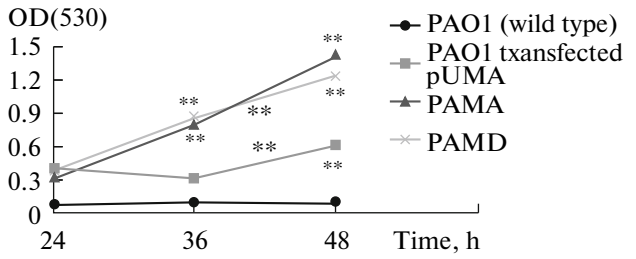

Fig. 5. Alginate production assays of 4 kinds of strains. Relative levels of alginate at each time point normalized by those of total protein of the same cell culture. Data represent the mean ± standard deviationfrom three independent experiments. $P < 0.05$, $P < 0.01$.

ACKNOWLEDGMENTS

We thank Weizhong Sun, Yingbin Zhou, Junming Zhu and Xiao D. Shen for their technical assistance duringexperimental work in this research. This work was supported by the National Natural Science Foundation of China (No. 30901593).

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