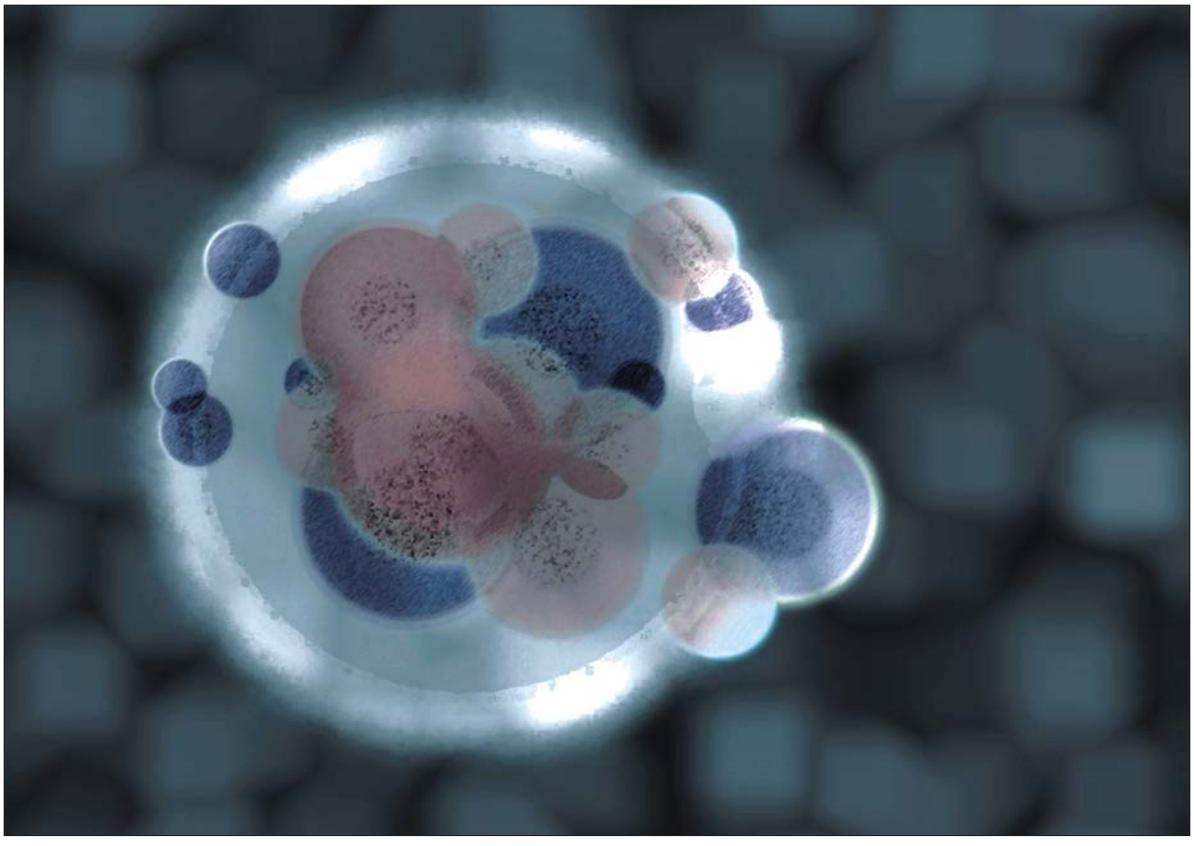

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 5 (2): 186-192, 2005
ISSN 1727-3048

Regulation of *toxA* by *ptxR* in the *Pseudomonas aeruginosa* Strain PA103ΔXR

Rukaia Mohammed Gashgari
Girl's Collage, P.O.Box 45057 Jeddah 21512, Saudi Arabia

Abstract: In this study we have examined *ptxR* expression in the *P. aeruginosa* strain (PA103ΔXR) which displays a unique phenotype. The strain was derived from PA103-2R which contains two copies of *ptxR* and was constructed by the integration of a *ptxR* plasmid in the PA103 chromosome. PA103ΔXR was isolated by continuously subculturing PA103-2R in antibiotic free media and screening for carbencillin sensitive colonies. Under iron deficient medium, *toxA* transcription is enhanced significantly. Two peaks of *toxA* transcription were detected at 4 and 16 h post inoculation. In contrast, *toxA* transcription in PA103ΔXR was significantly reduced. In iron-sufficient medium *toxA* transcription was repressed in both PA103 and PA103ΔXR. *ptxR* regulates *toxA* expression through the *regAB* locus. Under iron deficient conditions *regA* transcription was enhanced after 8 h of growth and reached a peak at 16 h of growth. This was followed by a sharp reduction in *regA* transcription during 20 and 24 h of growth In contrast, *regA* transcription in PA103ΔXR was significally reduced at 12 and 16 h of growth. Under high iron conditions, the patterns of *regA* transcription in PA103 and PA103ΔXR appears to be similar In both strains, it is significantly reduced. The unique phenotype of PA103ΔXR is not due to mutations in either *toxA*, *regAB* and *ptxR*. A plasmid carrying intact *toxA*, *regAB* and *ptxR* failed to complete the defect of PA103ΔXR in exotoxin A synthesis.

Key words: *Pseudomonas aeruginosa,* exotoxin A, *toxA, ptxR, regAB*

INTRODUCTION

Pseudomonas aeruginosa is a gram negative opportunistic pathogen that causes severe infections in hospitalized patients[1-5]. Among the different virulence factors produced by *Pseudomonas aeruginosa* toxin A, which is an ADP-ribosyl transferase enzyme. The production of exotoxin A in *Psudomonas aeruginosa* is a complicated process that involves several positive and negative regulatory genes[6].

The gene that encodes exotoxin A, *toxA* is negatively regulated by iron at the transcriptional level. In an iron-limited environment such as within the human body, *toxA* is transcribed, translated into exotoxin A and then secreted from the bacterial cell. Exposure of the eukaryotic cell to exotoxin A results in cell death and release of iron and nutrients which can be used by *Pseudomonas aeruginosa*.

We have previously described a *Pseudomonas aeruginosa* gene, *ptxR* that positively regulates the production of exotoxin A. In the presence of a *ptxR* plasmid, exotoxin A is increased by 3-4 fold in PA103 and we constructed a PA103-2R that contains two copies of *ptxR* which exotoxin A is increased by 10 fold. In addition exotoxin A synthesis was partially deregulated with respect to iron. The effect on exotoxin A synthesis occurred at the transcriptional level[3,4].

In the Present study we have constructed a *Pseudomonas aeruginosa* PA103 strain (PA103ΔXR). The strain was derived from PA103-2XR which contains two copies of *ptxR*. The integrated plasmid is segregated, the loss of the integrated plasmid would result in a phenotype that is similar to the original parent strain PA103.

MATERIALS AND METHODS

Bacterial strains and plasmids: The bacterial strains and plasmids used in this study are listed in Table 1.

Growth conditions: Growth conditions to *Pseudomonas aeruginosa* strains were basically the same as previously described[7]. Briefly the cultures were grown overnight in Luria Bertani medium (LB) at 37°C. An aliquot of the culture was pelleted, washed and resuspended in Chelex-treated trypticase soy broth dialysate (Difco Laboratories, Detroit, Mich.) to which 1% glycerol and 0.05 M monosodium glutamate was added (TSB-DC)[8]. Resuspended cells were subcultured in either TSB-DC,or TSB-DC containing iron (20 μg of Fe^{3+}/mL), to an optical density (OD^{540}) of 0.02 to 0.05. Carbenicillin was added at a concentration of 300 μg mL^{-1}. Cultures were grown at 32°C with maximum aeration. Throughout the growth cycle of each culture (24 h), samples were collected every 4 h for β-galactosidase assay.

Table 1: Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Source/Reference
Pseudomonas aeruginosa PA103	Prototroph hypertoxigenic strain	Liu [14]
PA103ΔXR	PA103-ΔXR construction from PA103-2XRcontaining two copies of *ptxR, ptxR* segregated from the chromosome and the resulting strain contains one copy of *ptxR*,Cb^R	This study
Plasmids pSW205	Cb^R, promoterless *lacZ* fusion vector, carrying the 1.8-Kbp Pst I stability fragment	Storey *et al.*[7]
pSW228	Ap^R *toxA-lacZ* fusion	
pJAC7-1	Cb^R Km^R, a recombinant plasmid of pKT230 and pUC19 carrying *ptxR* on a 2.1-Kbp *Kpn*I-Bg/II fragment	Hamood-*et al.*[15,16]
pJAC24	Cb^R, a *ptxR-lacZ* fusion in pSW205	Colmer and Hamood[4]
pRL88	Cb^R, a *regA-lacZ* fusion in pSW205	
pLAFR	Tc^R Km^R Inc^R lambda cos+broad-host-range cloning vector	Friedman *et al.*[17]

Abbreviations: Cb, Carbenicillin; Km, Kanamycin; Tc, tetracycline; ^{R}, resistance; Incp, incompatibility group.

β-Galactosidase assays: To determine the level of β-galactosidase activity, cells were grown in TSB-DC medium. At each 2 h in timepoint, 1 mL samples were harvested from each culture. The level of β-galactosidase activity in each sample was determined as previously described[9].

Exotoxin A assays: The isolates were grown in TSB-DC medium at 32°C with maximum aeration for 14-16 h. A1.0 mL sample of each culture was centrifuged and the supernatant was retained for the assays. The exotoxin A assay was performed as previously described by Ohman *et al.*[8]. Briefly, a 10 μL aliquot of 8 M urea and 2% dithiothreitol (DTT) was added to 10 μL of the supernatant (to activate exotoxin A) and the mixture was incubated at 25°C for 15 min. A 25 μL aliquot of wheat germ elongation-factor 2 and a 25 μL aliquot of reaction buffer (125 mM Tris-HCl, 100 mMDTT) were then added to the mixture. The ADP ribosyl transferase reaction was detected by the addition of 5 μL of [14C] NAD (500 mci/mmol; Dupont NEN). After 15 min of incubation at 25°C, the reaction was terminated by the addition of 10% trichloroacetic acid and the mixture was filtered on nitrocellulose filters. The filters were then washed and air dried and the amount of radioactivity on each filter was determined by a liquid scintillation counter (Packard Tri-carp 2100TR liquid scintillation analyzer).

Immunoblotting assays: The presence of exotoxin A proteins in the supernatant of the isolates was detected by immunoblotting experiments as previously described[10]. The cells were grown as described for the exotoxin A assays. Approximately 40-50 μg of protein from the supernatant fraction was separated using 10% SDS-polyacrylamide gels (SDS-PAGE)[11]. The proteins were then transferred onto nitrocellulose membranes as described by Towbin *et al.*[12]. The membranes were blocked with 5% Blotto 5% nonfat dry milk in Towbin's saline (150 mM NaCl, 50 mM Tris-HCl, pH 7.5) and then exotoxin A rabbit polyclonal antibodies were added and the membranes were incubated for 2 h at 37°C. The membranes were then washed in Towbin's saline, reacted with goat anti-rabbit antibody labeled with horseradish peroxides (Sigma Chemical Co., St. Louis, MO) and developed with hydrogen peroxide and 4-chloro-1-napthol (Sigma).

The conjugative assay: A pLAFR *regA* was transferred to PA103 and Pa103Δ XR by triparental mating using the conjugative plasmid mm 294/pRK2013 as a helper[13].

RESULTS

Construction of PA103Δ *ptxR* strain: The strain was derived from another recently constructed strain PA103-2R. PA103-2R was constructed by the integration of a *ptxR* plasmid in the PA103 chromosome. This was done in an attempt to produce a strain that carries only two copies of *ptxR*. Such a strain would be preferred for different regulatory studies. However, the phenotype of PA103-2R was unique and unexpected. Exotoxin A synthesis in iron deficient medium increased by about 10 fold. The was significantly higher than the 4-5 fold increase that we usually detect in PA103 strain that contains multiple copies of *ptxR*. In addition exotoxin A synthesis was partially deregulated with respect to iron. The effect on exotoxin A synthesis occurred at the transcriptional level (data not shown).

To understand the mechanism that results in the unique phenotype of PA103-2R, we tried to isolate a derivative of PA103-2R from which the integrated plasmid is segregated. The main concept in this approach is that if the integration of the plasmid resulted in the phenotype, the loss of the integrated plasmid would result in a phenotype that is similar to the original parent strain PA103. Therefore strain PA103-2R was sub cultured extensively in LB broth that contains no antibiotic (the presence of an antibiotic is usually required to the maintain the plasmid in the cells). At the end of the eight subcultures, cells were plated on LB agar to obtain individual colonies. All isolated colonies were screened for a carbenicillin sensitive phenotype. One colony was isolated and designated PA103ΔXR. The loss of the

integrated plasmid from the chromosome of in PA103-ΔXRwas confirmed by Southern blot hybridization experiments. Chromosomal DNA was obtained from the strains PA103, PA103-2R and PA103 ΔXR. The DNA was digested with Hind III restriction enzyme and the digested DNA was transferred to a nylon membrane. The membrane was hybridized with two separate probes; vector probe and a *ptxR* probe. Hybridization analysis with the vector probe revealed the presence of pUC18 DNA in the chromosome of PA 103-2R only (data not shown). No vector DNA was detected within the chromosome of PA103 or PA103ΔXR (data not shown). Hybridization with the *ptxR* probe revealed the presence of two copies of *ptxR* within the chromosome of PA103-2R but a single copy of *ptxR* with the chromosome of PA103 and PA103ΔXR (data not shown).

These results indicate that the integrated *ptxR* plasmid had segregated from the chromosome of PA103-2R and the resulting strains contains only one copy of *ptxR*. Whether other DNA rearrangements occurred during the segregation of plasmid is not known at this time.

Growth characteristics of the PA103ΔXR: It is possible that the mutation in PA103ΔXR affected its growth. To examine this possibility we have compared the growth of PA103ΔXR with its parent strain PA103 throughout their growth cycles. Cells were inoculated into TSB-DC medium, to an OD^{540} of about 0.03 and samples were obtained every 2 h for 24 h. As shown in Fig. 1 and 2, the growth pattern of PA103ΔXR is similar to that of PA103 under both iron deficient and iron sufficient conditions suggesting that the mutation caused no change in metabolic characteristics of the strain.

The effect of the *ptxR* on exotoxin A transcription: We have previously shown that *ptxR* enhances exotoxin A synthesis in *Pseudomonas aeruginosa* by 4 fold. The parent strain of PA103ΔXR contained 2 copies of *ptxR*. In that strain exotoxin A synthesis was significantly enhanced and deregulated with respect to iron. In addition, the production of the pyoverdine chromophore was also deregulated with respect to iron. Furthermore, the strain produced increased level of the type III effectors molecule ExoU. Strain PA103ΔXR carries only one copy of *ptxR*. However, is not known if the gene is intact or if there are other DNA rearrangements within the sequences surrounding the gene.

In our analysis of the strain we tried to determine the level of exotoxin A transcription throughout the growth cycle of the strain and compare that with its parent strain PA103. To examine *toxA* transcription, we have utilized

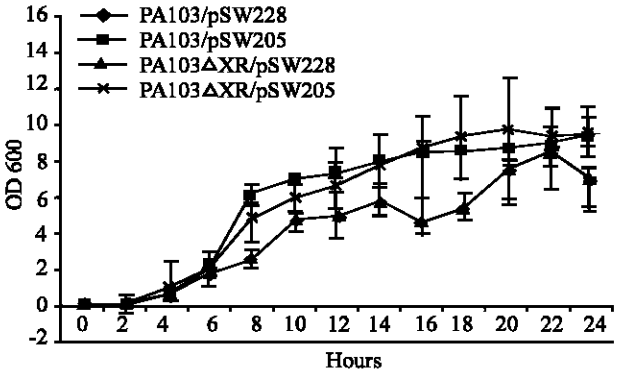

Fig. 1: Comparison of growth rate of PA103 and *toxA-lacZ* fusion (pSw228) in iron deficient medium (Values represent three independent experiments)

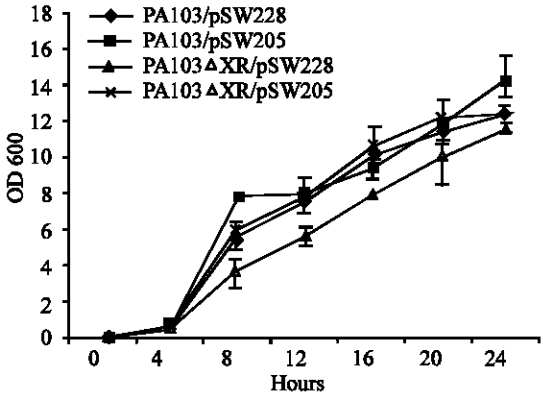

Fig. 2: Comparison of growth rate of PA103 and *toxA-lacZ* fusion (pSw228) in iron sufficient medium (Values represent three independent experiments)

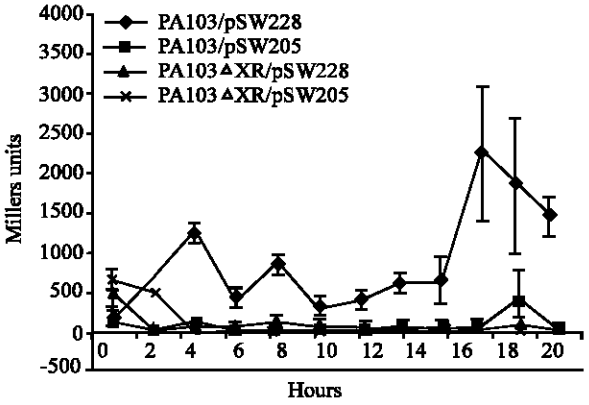

Fig. 3: The expression of the *toxA* gene throughout the growth cycle of the strains in iron deficient medium (values represent three independent experiments)

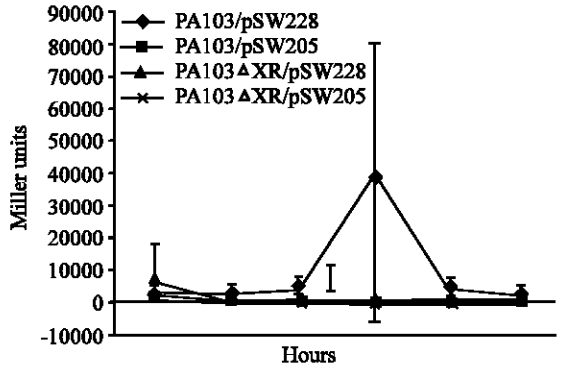

Fig. 4: The expression of the *toxA* gene throughout the growth cycle of the strains in iron sufficient medium (values represent three independent experiments)

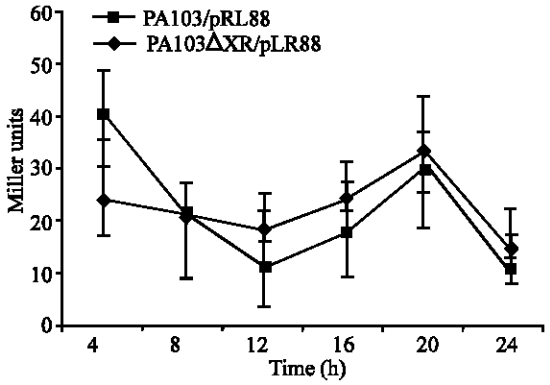

Fig. 5: The levels of *regAB* transcription within PA103ΔXR and its parents PA103 using the *regA-LacZ* translational fusion plasmid pRL88 in iron deficient medium

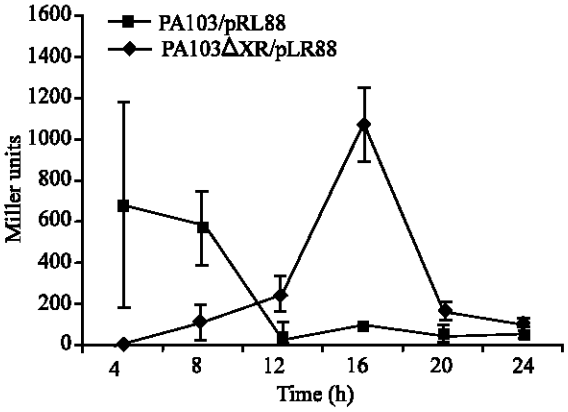

Fig. 6: The levels of *regAB* transcription within PA103ΔXR and its parents PA103 using the *regA-LacZ* translational fusion plasmid pRL88 in iron sufficient medium

plasmid pSW228 which carries a *toxA –lac Z* translational fusion. In this plasmid the *toxA* upstream region plus the region that codes for the first 12 amino acids of exotoxin was fused to the β-galactosidase gene as previously described.

Cells were grown either in TSB-DC or in TSB-DC with iron and samples were obtained every 2 h. The level of β-galactosidase activity in the lysate fraction was determined as previously described. As shown in Fig. 3, under iron deficient medium, *tox A* transcription is enhanced significantly. Two peaks of *toxA* transcription were detected at 4 and 16 h post inoculation. In contrast, *toxA* transcription in PA103ΔXR was significantly reduced (Fig. 3). The typical pattern of *toxA* expression when *P. aeruginosa* was grown in iron-sufficient medium was not detected (Fig. 3). In iron-sufficient medium *toxA* transcription was repressed in both PA103 and PA103ΔXR (Fig. 4). These results suggest that the mutation in PA103ΔXR altered *tox A* transcription significantly.

The effect of *ptxR* on the expression of the *toxA* regulatory gene *regAB*: Previous studies have shown that *ptxR* regulates *toxA* expression through the *reg AB* locus. The presence of a *ptxR* plasmid within a *Pseudomonas aeruginosa* strain that carries a deletion within the *regAB* locus had no effect on *toxA* expression. In addition the presence of *ptxR* plasmid in *Pseudomonas aeruginosa* enhances *regAB* transcription byfold. Therefore we tried to determine if the effect on exotoxinA synthesis in PA103ΔXR involves the *reg AB* locus. This was done by determining the level of *reg AB* transcription within PA103ΔXR and its parent strain, PA103, using the *reg AB-lacZ* translational fusion plasmid pRL88. In pRL88, the *regA* upstream region plus the region that codes for the first few amino acids of *regA* protein was fused in frame with the *lacZ* gene. PA103/pRL88 and PA103ΔXR/pRL88 were grown in TSB-DC in the presence and absence of iron and the level of β-galactosidase activity was determined as previously described. Under iron deficient conditions *regA* transcription was enhanced after 8 h of the growth and reached a peak at 16 h of growth (Fig. 5). This was followed by a sharp reduction in *regA* transcription during 20 and 24 h of growth (Fig. 5). In contrast, *regA* transcription in PA103ΔXR was significally reduced at 12 and 16 h of growth (Fig. 5). Under high iron conditions, the patterns of *regA* transcription in PA103 and PA103ΔXR appear to be similar (Fig. 6). In both strains it is significantly reduced (Fig. 6). However even the reduced level of transcription in PA103ΔXR followed

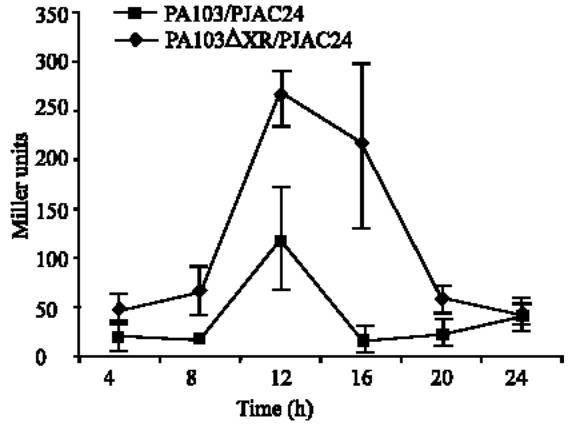

Fig. 7: *ptxR* expressions using plasmid pJA24 within the strains that were grow in iron difficult medium (Values represent three independent experiments)

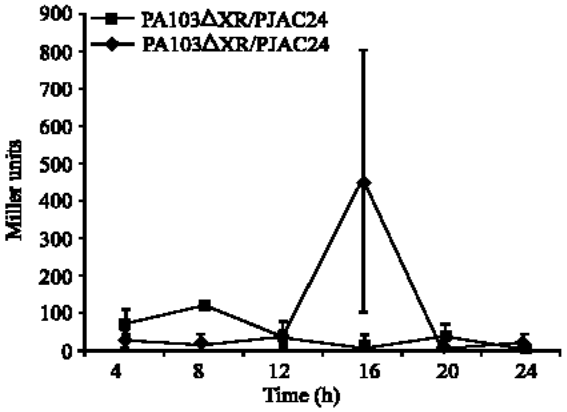

Fig. 8: *ptxR* expressions using plasmid pJA24 within the strains that were grow in iron difficult medium (Values represent three independent experiments)

a unique pattern. An initial reduced level at 12 h of growth was followed by a sharp decline at 24 h of growth (Fig. 6).

The effect of *ptxR* on *ptxR* expressions: *ptxR* belong to the *LysR* family of transcriptional activators. One of the characteristic features of this family is that the proteins autoregulates their own synthesis. We don't know if *ptxR* autoregulates it own synthesis at this time. We tried to determine if the mutation in the strain affects *ptxR* expression or the pattern of *ptxR* expression. Based on previous studies *ptxR* expression may not be as stringently controlled by iron as *regA* or *toxA*. Therefore, we examined *ptxR* expression under iron deficient conditions only. This was done using plasmid pJAC24 in which the *ptxR* upstream region plus the region that codes for the first 55 amino acides of *ptxR* protein was fused in frame with the β-galactosidase gene. Cells were

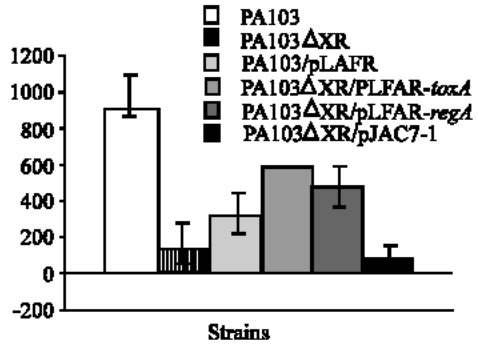

Fig. 9: The level od ADP-ribsoyl transferase activity produced by PA103ΔXR carrying *toxA*, *regAB* or *ptxR* or were introduced into PA103ΔXR by electroporation (Complemention analysis)

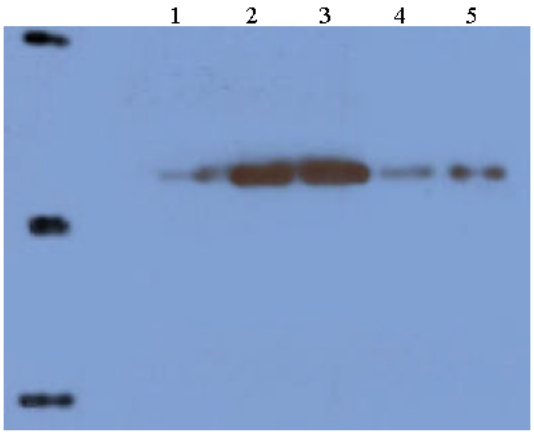

Fig. 10: Immunblot analysis of PA103Δ
1. PA103ΔXR
2. PA103/pLAFR
3. PA103ΔXR/pLAFR-*tox A*
4. PA103ΔXR/pLAFR-*reg A*
5. PA103ΔXR/pJAC7-1

grown under iron deficient conditions and the level of β-galactosidase activity was determined as previously described. As shown in PA103, *ptxR* transcription followed a characteristic pattern (Fig. 7). The initial increase in *ptxR* transcription was detected at 4 h of growth (Fig. 7). This were followed by a peak at 12 h of growth, then the level of transcription declined gradually towards 20 h of growth (Fig. 7), in PA103ΔXR, the pattern of *ptxR* transcription was very similar to that in PA103 at several time points (Fig. 7). At 16 h of growth the reduction in the level of *ptxR* transcription is more than 20 fold (Fig. 7). Under high iron condition, the patterns of *ptxR* in PA103 and PA103ΔXR appear to be similar (Fig. 8). In both strains is significantly reduced

(Fig. 8). However even the reduced level of transcription in PA103ΔXR followed a unique pattern.

Complementation analysis of PA103ΔXR: We tried to determine if the defect in PA103ΔXR resides within *toxA*, *regAB* and *ptxR*. Therefore, we examined the ability of plasmids that carried intact *toxA, regAB* and *ptxR* to complement the defect of PA103ΔXR in toxin A synthesis. The plasmids were introduced into PA103Δ *ptxR* by electroporation. Cells were grown in iron deficient media and the level of *toxA* activity was determined as previously described. As shown in Fig. 9 the presence of either pLAFR-*toxA*, pLAFR-*regA* and pJAC7-1 had no effect on toxin A synthesis. PA103ΔXR is not defective in any of the three genes. This segregation was further confirmed by immunbloting analysis using toxinA antibodies (Fig. 10).

DISCUSSION

Present results showed that strain PA103ΔXR has a unique phenotype. The strain was defective in the expression of *ptxR, regA* and *toxA* (Fig. 9). It is clear that the strain is not defective in *toxA, reg A* and *ptxR*. Complementation analysis, using plasmids that carry intact genes, showed that none of the plasmids complemented the defect in the mutant. The regulation of *toxA* expression is multilayered and involves several regulators. For example, the *regAB* locus positively regulates *toxA* expression. In the presence of a *regA* plasmid, *toxA* expression in *Pseudomonas aeruginosa* is increased by about 10 fold. In addition *Psudomonas aeruginosa* strains that carry mutations within the *regA* gene, produced neither exotoxin A protein nor *toxA* transcript. Both *toxA* and *regAB* are regulated by iron, (their expression is inhibited when *Pseudomonas aeruginosa* is grown in iron sufficient medium). The expression of *toxA* and *regA* is regulated by *pvdS*, which is an alternative sigma factor[7]. Finally, Further ferric uptake regulator and represes the expression of *pvdS* which can no longer enhance the expression of *regAB* and *toxA*.

Therefore, based on these findings, it is likely that the mutantion in PA103ΔXR occurred in a gene that regulates the expression of *regAB, ptxR* and *toxA*[4,5]. One possible approach to identify the mutation is to conduct additional complementation experiments using the *pvdS* gene. This will help us exclude the possibility that the strain is defective in *pvdS*. Preliminary experiments, however do not support this possibility. Since *pvdS* regulates the expression of both *toxA* and the siderophore, pyoverdine, we examined PA103ΔXR pyoveridine synthesis. Unlike the situation with exotoxin A, pyoverdine production was not altered (data not shown). Therefore the defect may occur in a genes that regulate *pvdS*. To identify such a gene, it is important to define the exact nature of the mutation in this strain. The would include the mobilization of a *Pseudomonas aeruginosa* gene bank into the strain and examine the individual colonies for the production of exotoxin A.

One of the puzzling findings in this study is that the strain may carry a mutation in gene that is not yet identified. This strain was not produced by any known mutagenesis such as transposn mutagenesis. It was not produced even by the gene replacement technique. Its parent strain carried two functional copies of *ptxR*. This was confirmed by southern blot hybridization analysis. This strain PA103ΔXR was derived from PA103 2R by simply growing the culture in the absence of antibiotic and allowing the plasmid to segregate out. Strain PA103ΔXR carries only one copy of *ptxR*. Therefore, we expected the change to include possible rearrangement within the vicinity of *ptxR* or even within *ptxR*. However this assumption is not supported by experimental data. Based on these findings it, would be essential to identify the gene whose defect resulted in this phenotype. This will help us understand further the complicated cycle of *toxA* regulation in *Pseudomonas aeruginosa*.

ACKNOWLEDGMENTS

The author wish to thank Dr. Abdul Hamood, Department of Microbiology Texas Tech. Univ., USA, Health Since Center, for his kind supervision and great help in all of this work, Dr. Jane Colmer and Dr. Nancy Carty for technical assistance of experiments and reviewing the manuscript.

REFERENCES

1. Bergen, G.A. and J.H. Shelhamer, 1996. Pulmonary infiltrates in the cancer patient. New approaches to an old problem. Infect. Dis. Clin. North Am., 10: 297-325.
2. Rumbaugh, K., J.A. Griswold and A.N. Hamood, 1999a. *Pseudomonas aeruginosa* strains obtind from patients with tracheal, urinary tract and wound infection: Variations in virulence factors and virulence genes. J. Hospital Infect., 43: 211-218.
3. Rumbaugh, K.P., A.N. Hamood and J.A. Griswold, 1999b. Anaysis of *Pseudomonas aeruginosa* clinical isolates for possible variations within the virulence genes exotoxin A and exoenzyme S. J. Syrgical Res., 82: 95-105.

4. Colmer, J.A. and A.N. Hamood, 1998. Characterization of *ptxS*, a *Pseudomonas aeruginosa* gene which interferes with the effect of the exotoxin A positive regulatory gene, *ptxR*. Mol. Gen. Genet., 258: 250-259.
5. Colmer, J.A. and A.N. Hamood, 2001, Molecular analysis of the *Pseudomonas aeruginosa* regulatory genes *ptxR* and *ptxS*. Can. J. Microbiol., 47: 820-828.
6. Sawanson, B. and A.N. Hamood, 2000. Autoregulation of *Pseudomonas aeruginosa protein PtxS* occurs through a specific operator site within the *ptxS* upstream region. J. Bacteriol., 182: 4366-4371.
7. Storey, D., T. Raivio, D. Frank, M.J. Wick, S. Kaye and B.H. Iglewski, 1991. Effect of *regB* on expression from the P1 and P2 promoters of the *Pseudomonas aeruginosa regAB* operon. J. Bacteriol., 173: 6088-6094.
8. Ohman, D.E., J.C. Sadoff and B.H. Iglewski, 1980. Toxin A-deficient mutants of *Pseudomonas aerginosa* PA103: Isolation and characterization. Infect. Immun., 28:899-908.
9. Stachel, S.E., G. An, C. Flores and E.W. Nester, 1985. ATn3 *lacZ* transposon for the random generation of β-galactosidase gene fusions: Application to the analysis of gene expression in *Agrobacterium*. The EMBO J., 4: 891.
10. Hamood, A.N., M.J. Wick and N.H. Iglewski, 1990. Secretion of toxin A from *Psudomonas aeruginosa* PAO1, PKA and PA103 by *Escherichia coli*. Infect. Immun., 58: 1133.
11. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 227: 680.
12. Towbin, H., T. Staehelin and J. Gordon, 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci.,76: 4350-4354.
13. Ditta,G., S. Starfield, D. Corbin and D.R. Helinski, 1980. Board host range DNA cloning system for gram negative bacteria. Construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA., 77: 7347-7351.
14. Liu, P.V., 1973. Exotoxin A of *Pseudomonas aeruginosa*. Factors that influence the production of exotoxin A. J. Infect. Dis., 128: 506-513.
15. Hamood, A.N., J.A. Griswold, C.M. Duhan, 1996. Production of extracellular virulence factors by *Pseudomonas aeruinosa* isolates from tracheal, urinary tract and wound infections. J. Surgical. Res., 61: 425-432.
16. Hamood, A.N., J.A. Griswold and J. Colmer, 1996. Characterization of elastase–deficient clinical isolates of *Pseudomonas aerginosa*. Infect. Immun., 64: 3154-3160.
17. Friedman, A.M., S.R. Brown, W.J. Buikkema and F.M. Ausubel, 1982. Construction of a braid host cosmid cloning vector and its usage in the genetic analysis of Rhizobium mutants. Genetics, 18: 289-296.