Nucleotide sequence and characterization of *toxR*: a gene involved in exotoxin A regulation in *Pseudomonas aeruginosa*

D.J.Wozniak, D.C.Cram, C.J.Daniels and D.R.Galloway*

Department of Microbiology, The Ohio State University, Columbus, OH 43210, USA

Received September 10, 1986; Revised and Accepted January 27, 1987

ABSTRACT

We have previously reported (1) the discovery and subsequent cloning of a regulatory gene, designated *toxR*, which appears to regulate the expression of the exotoxin A (ETA) structural gene *toxA*. Subsequent work by this laboratory has resulted in the subcloning of the *toxR* gene and its transfer to a high copy number plasmid (pGW28). Functional analysis of the *toxR* gene using a Tn5 insertion along with *toxR* deletions indicates that inactivation of *toxR* results in a dramatic reduction of ETA production. Nucleotide sequence analysis of pGW28 has revealed a 675 bp major open reading frame (225 codons) which could encode for a protein of 24,626 daltons. Using S1 nuclease mapping, the *toxR* RNA transcript has been shown to originate 20 bp upstream of the presumptive translation initiation codon. Experiments using a *toxA* specific probe have revealed the the *toxR* gene product appears to regulate the expression of ETA at the transcriptional level.

INTRODUCTION

Exotoxin A (ETA) is a potent bacterial exotoxin which is produced as a single chain protein by greater than 90% of all *P. aeruginosa* strains (2). ETA, with a molecular weight of 66,583 daltons, is a member of the ADP ribosyltransferase family of bacterial toxins and is capable of inactivating eucaryotic protein synthesis by covalently modifying elongation factor two (EF-2) through the attachment of an ADP-ribosyl moiety obtained from NAD (3). The biochemistry of ETA has been well studied, including a recent description of the three-dimensional configuration of the molecule at the 3 Å level of resolution (4). However, details concerning the genetic regulation of this protein have remained somewhat obscure. The structural gene for ETA (*toxA*) has been cloned (5-6) and it is widely reported that expression of *toxA* is dependent upon the level of iron in the culture media (7). It has recently been suggested that iron affects the expression of *toxA* at the transcriptional level (8).

We have previously described the isolation of a regulatory gene in *P. aeruginosa* which appears to control the expression of *toxA* in a positive

manner (1). This gene, now designated toxR, appears to encode for a trans-acting product which increases the expression of ETA approximately 10-fold in all toxigenic P. aeruginosa strains tested (1). In this paper, we describe the subcloning of the toxR gene and the determination of its nucleotide sequence. Furthermore, we demonstrate that the toxR gene product is required for efficient expression of the toxA gene and that it appears to act as a positive regulator at the transcriptional level.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The P. aeruginosa strains used in this study are PA103 (9) and PA103-29 (10). All pUC18 (11) based recombinant plasmids were transformed into strain TB1. Similarly, E. coli HB101 (12) or P. aeruginosa strains were used for transformations involving the broad host range plasmid pR01614 (13). pR01614 was used as a shuttle vector since it contained unique EcoRI and HindIII restriction sites into which cloned DNA fragments containing toxR sequences could be inserted.

Media and general procedures.

Bacteria were grown at 37°C in LB medium for routine genetic manipulations. The dialysate of trypticase soy broth (TSBD) was used to culture bacteria in which levels of ETA were analyzed (14). Antibiotic concentrations for plasmid selection in P. aeruginosa were 300 µg/ml carbenicillin and 100 µg/ml tetracycline, while those for selection in E. coli were 100 µg/ml carbenicillin or ampicillin and 15 µg/ml tetracycline. Plasmid DNA was isolated from strains of E. coli by the method of Birnboim and Doly (15) and purified by centrifugation in cesium chloride-ethidium bromide gradients. RNA was extracted from late-log (O.D.₅₈₀ = 0.8) P. aeruginosa cultures by either the hot-phenol method (16) or by centrifugation in cesium chloride in the presence of guanidine isothiocyanate (17). Restriction endonuclease digestion of DNA, agarose and acrylamide gel electrophoresis, ligations and transformations involving E. coli were performed essentially as described by Maniatis, et al. (17). The method of Olsen, et al. (13) was used to transform P. aeruginosa with plasmid DNA. Tn5 mutagenesis was performed according to the protocol of deBruijn and Lupski (18). Conditions for hybridization of end-labeled DNA to RNA immobilized on nylon membrane were essentially those recommended by Biorad.

DNA sequencing.

Nucleotide sequence analysis of DNA was performed by the chemical

cleavage method described by Maxam and Gilbert (19) with minor adjustments. The G+A base modification reaction was incubated at 37°C for 10 min. while the G, T+C and C base modification reactions were incubated at 0°C for 10 min. S1 nuclease mapping.

The toxR transcription initiation site was determined essentially by the method outlined in Maniatis et al. (17). RNA was isolated from PA103-29/pGW28 cells grown to late log phase (O.D.₅₈₀ = 1.0). DNA probes 5' end labelled specifically on the coding strand of the toxR gene were mixed with cellular RNA in a 30 µl volume, heated at 85°C for 5 min. and hybridized at 56°C. After 14 hours incubation, 300 µl of S1 nuclease buffer containing 100 units of S1 nuclease (Boehringer Mannheim) was added and allowed to incubate at 37°C for different time intervals. The S1 nuclease digestion products were then resolved on a 6% polyacrylamide sequencing gel.

Detection of ETA.

Levels of ETA were measured by either the adenosine diphosphate ribosyltransferase (ADPR) assay (14) or by solid phase radioimmunoassay (SPRIA) as previously reported (1,20). In both methods, Lowry protein determinations were performed on the test culture supernatants in order to normalize ETA levels.

RESULTS AND DISCUSSION

toxR subcloning and phenotypic analysis.

Initially, the 3.1 kb XhoI fragment from pFHK10 containing toxR (1) was inserted into the unique SalI site in the polylinker of pUC18 generating the plasmid pGW01 (Fig. 1). Dissection of pGW01 with an array of restriction endonucleases enabled us to formulate an extensive restriction map of the 3.1 kb region (Fig. 2). In order to subclone various fragments from pGW01, the multiple cloning sites of its vector moiety together with restriction sites within the 3.1 kb insert were utilized. By employing this strategy, a series of pUC18 derived subclones were subsequently generated. To determine the toxR phenotypes, it was necessary to reclone the inserted fragments individually into the broad host range plasmid pR01614 (13), transform them into the phenotypically Tox⁻ strain P. aeruginosa 103-29, and assay for levels of ETA. The appropriate subcloned regions of the pUC18 recombinant plasmids were simply removed by EcoRI and HindIII cleavage and inserted into similarly digested pR01614; an example of this strategy is shown in Fig. 1.

Phenotypic analysis (ADPR activity and SPRIA) showed that except for pGW21, only pGW28 containing cells produced elevated levels of ETA; none of

Fig. 1. Subcloning strategy. The construction of pFHK10 has been described previously (1). Filled segments represent cloned regions, while thin lines are vector sequences. Antibiotic resistance designations are indicated along with respective plasmid sizes.

the other plasmids significantly stimulated ETA synthesis. Immunoblots of the culture supernatants from these strains confirmed these observations (Fig. 3). On the basis of these results, the *toxR* gene must be contained entirely within the 1.9 kb *XhoI*-*PstI* fragment (Fig. 2). In addition, since pGW23 (*SacI* fragment) and pGW29 (*SacI* Δ) do not contain *toxR* entirely (Fig. 2), the *toxR* gene must span the unique *SacI* site. Furthermore, the *BamHI* site must also be located in the *toxR* gene since both pGW26 (*BamHI* fragment) and pGW25 (*BamHI* Δ) fail to increase ETA expression in transformed hosts (Fig. 2).

Fig. 2. Restriction map and subclones of the 3.1 kb region. Open and filled segments represent pR01614 and potential *toxR* regions, respectively. Corresponding insert sizes are designated below each fragment. Following transformation of each plasmid into strain PA103-29, ETA levels are expressed as (a) ADPR assay in cpm of enzyme activity per μ g of culture supernatant protein or (b) SPRIA in ng of ETA per μ g of culture supernatant protein. The arrow indicates the position of a Tn5 transposon insertion. Only pertinent restriction sites are indicated.

In order to further define the limits of *toxR*, transposon Tn5 mutagenesis of pGW21 was undertaken. Of numerous insertions generated, only 1 mapped within the 3.1 kb fragment, 130 bp left of the *SacI* site (Fig. 2); the remainder mapped in the vector moiety of pGW21. Cells containing this plasmid, pGW21::Tn5, did not produce significant levels of ETA (Figs. 2,3 lane I) indicating that the Tn5 insertion interrupted natural expression of *toxR*.

Nucleotide sequence of the *toxR* gene.

The nucleotide sequence of approximately 900 bp containing the *toxR* gene was determined by sequencing both strands as outlined in Fig. 4. Computer analysis of the sequence data revealed a major open reading frame (ORF) (225 codons, Fig. 5). We employed the codon usage evaluation program of Stadin and McLachlan (21) as well as the RNY codon rule (22) and concluded that only the

Fig. 3. Immunoblot analysis. Culture supernatants from strains grown overnight in TSBD medium were electrophoretically separated on a 10% SDS polyacrylamide gel, transferred to nitrocellulose and ETA identified using immunoaffinity purified antitoxin (20) and ^{125}I protein A. Lane assignments are as follows:
 A. Purified ETA; B. PA103-29/pR01614; C. PA103-29/pGW21;
 D. PA103-29/pGW25; E. PA103-29/pGW26; F. PA103-29/pGW27;
 G. PA103-29/pGW28; H. PA103-29/pGW29; I. PA103-29/pGW21::Tn5;
 J. PA103-29.

major ORF initiating at position 169 with an AUG start codon and terminating at position 841 with a UGA stop codon, was likely to encode for a protein. This ORF spans both the SacI and BamHI restriction sites as predicted from phenotypic analysis of the subclones and would code for a polypeptide of

Fig. 4. Maxam - Gilbert DNA sequencing strategy. Large block indicates extents of the *toxR* gene. Restriction sites are indicated in order. Filled dots below particular sites designate those sites which were 5' end labeled using γ ^{32}P ATP in the presence of T4 polynucleotide kinase. Arrows represent the sequencing limits accomplished for each end labeled fragment.

Fig. 5. Nucleotide sequence of the non-coding strand of the *toxR* gene. Nucleotides are numbered 1 to 961. Pertinent restriction endonuclease sites are shown in brackets. The boxed region indicates a potential RBS. The vertical arrow represents the transcription initiation origin as defined by S1 nuclease mapping. Horizontal arrows are regions of dyad symmetry following the translation termination codon (asterisk) which are characteristic of *rho* independent transcription terminators.

24,626 daltons. Codon usage in this ORF (Table 1) showed that there is a strong preference for codons with G or C at the first and third positions (73% and 80% respectively), a feature which has been observed in other *P. aeruginosa* genes (5,23,24). In addition, a significant degree of correlation was noted between codons used in the *toxA* gene (5) and *toxR* gene (data not shown). Codons GAC, CGC, CUG, GGC, and GCC are frequently used whereas GUU, UUA, UCU, UGU, and UCA are not used at all.

Analysis of the upstream sequences of this ORF reveals several interesting features. Nine bp upstream from the AUG initiation codon lies a sequence 5' AGAG 3' which weakly resembles an *E. coli* ribosomal binding site

TABLE 1. CODON USAGE OF toxR

THIRD BASE					THIRD BASE				
	U	C	G	A		U	C	G	A
UC	0	3	3	0	UU	0	5	4	0
CC	1	4	6	2	CU	3	10	20	1
AC	2	2	2	2	AU	1	5	1	0
GC	5	12	11	1	GU	0	6	2	0
TOTAL	8	21	22	5	TOTAL	4	26	27	1
UG	0	6	1	1	UA	1	2	0	0
CG	4	11	4	1	CA	4	5	7	4
AG	0	4	3	1	AA	0	7	4	1
GG	1	12	1	2	GA	4	9	6	5
TOTAL	5	33	9	5	TOTAL	9	23	17	10

Numbers indicate the frequency that a particular codon is used in the toxR gene. Overall GC content is 66.4%; first base 73%, second base 48%, third base 80%.

(RBS) (Fig. 5). Another sequence 5' GAGCCATC 3' which includes 3 bp of the putative RBS shares complete homology with a sequence located in approximately the same upstream position of the toxA gene (5) and, with one change, in the genes encoding tryptophan synthase from *P. aeruginosa* (23). Further upstream at position 58 is a sequence which resembles conserved dodecamers that are also present in both the toxA gene (5) and a gene encoding phospholipase C in *P. aeruginosa* (24). Downstream of the ORF are inverted repeats which could be involved in transcription termination of toxR.

Further analysis of the sequence data revealed a minor ORF (174 codons) which initiates at position 228 with an AUG and terminates at position 750 with a UGA (Fig. 5) and thus lies within the major 225 codon ORF. This minor ORF was not supported by either the codon usage evaluation program (21) or the RNY codon rule (22).

Fig. 6. S1 nuclease mapping of the 5' end of the *toxR* transcript. Lanes G, G+A, C+T and C represent a sequence ladder of the 550 bp *Sal*I-*Eco*RV fragment used for S1 mapping (Fig. 2). Lanes 1,2,3 and 4 are products obtained from S1 nuclease digestion: Lanes 1,2, and 3; RNA and DNA probe mixtures incubated with S1 nuclease for 15, 10 and 5 min., respectively. Lane 4; DNA probe incubated with S1 nuclease for 15 min. Lane 5; DNA probe minus S1 nuclease. The arrow represents the major DNA fragment protected from S1 nuclease digestion.

Moreover, this minor ORF lacked sequences normally involved in the regulation of prokaryotic genes including a RBS and a potential transcription terminator. In addition, little correlation of codon usage was noted between this minor ORF and the *toxA* gene (5). Based on these observations, it is unlikely that the minor ORF encodes the *toxR* activity.

ToxR S1 nuclease protection analysis.

In an S1 nuclease protection experiment, a 550 bp *Sal*I-*Eco*RV fragment (Fig. 2), 5' end labelled on the coding strand, was used to hybridize to pGW28 specific RNA. After S1 nuclease digestion, a 180 bp DNA fragment was protected (Fig. 6, lanes 1,2, and 3). The 550 bp *Sal*I-*Eco*RV probe alone,

Fig. 7. DNA-RNA hybridizations. Total cellular RNA was extracted from PA 103-29 harboring plasmids; (b) pR01614; or (c) pGW28 (toxR). An 820 bp SalI fragment contained from within the coding region of toxA was 5' end labeled and used as a toxA specific probe. RNA quantities were adjusted so that equal amounts were applied to the filter. As a control, (a) ptox DNA (5) was denatured, filtered and probed along with RNA. A laser densitometry scan of the resulting autoradiography is shown below the figure.

either treated with S1 nuclease (Fig. 6, lane 4) or left untreated (Fig. 6, lane 5), produced no comigrating 180 bp fragment. On the basis of these results, transcription of toxR initiates at the adenine residue 20 bp upstream of the presumptive toxR translation initiation codon (Fig. 5). Sequences resembling E. coli consensus -10 and -35 regions are not evident upstream from this S1 site and this may account for the fact that we have not detected the production of a 24,626 dalton toxR protein in E. coli minicells carrying pGW28.

RNA blot analysis of toxA mRNA production.

In order to determine whether toxR affects the level of toxA mRNA, we performed RNA blot analysis of mRNA produced by a phenotypically non-toxicogenic strain, PA103-29/pR01614, and PA103-29/pGW28, a toxR recombinant strain with a restored ETA phenotype. The results shown in Figure 7 demonstrate that the Tox⁻ mutant PA103-29/pR01614 produces no detectable RNA capable of binding to a toxA specific gene probe (File B). However, the toxR-transformed strain PA103-29/pGW28 produces an elevated amount of RNA which specifically

hybridizes with the toxA probe (File C). This result suggests that the toxR gene product may act at the transcriptional level of the toxA gene. In addition, RNA blot analysis of strains containing a Tn5 transposon inserted 47 bp upstream of the toxR gene (Fig. 2) revealed that no RNA capable of hybridizing to the toxA probe is produced (data not shown). This suggests that P. aeruginosa strain 103-29 contains a mutation in the toxR gene and offers further evidence in support of a requirement for a positive regulatory element which is essential for high expression of the toxA gene.

CONCLUSION

The results presented in this paper describe the physical characterization of a gene (toxR) involved in the positive regulation of the exotoxin A gene (toxA) in P. aeruginosa. Sequence determination of toxR, including upstream region analysis, has revealed a large ORF (225 codons) capable of encoding a 24,626 dalton protein. Codon usage is non-random and appears to be closely related to the frequencies found in P. aeruginosa for the toxA gene (5). Overall, the toxR gene consists of a 66.4% G + C content which is similar to the toxA gene. Examination of the upstream sequence of toxR reveals the lack of consensus E. coli transcriptional start signals, analogous to what has been described for the toxA gene (5). It is interesting to note that some Pseudomonas genes are efficiently expressed in E. coli, for example pilin genes (25), and this could be due to the presence of promoter regions resembling typical E. coli -10 and -35 sequences. In contrast, other P. aeruginosa genes, including toxA (5,6) are poorly or not at all expressed in E. coli unless placed under the control of a strong E. coli promoter.

We have also demonstrated that toxR is required for the efficient expression of toxA; when toxR is inactivated by transposon mutagenesis or by partial deletion, ETA levels are significantly reduced or eliminated. The ability to restore a Tox⁺ phenotype to P. aeruginosa strain 103-29 (1) by transformation with toxR and the lack of toxA-specific mRNA production in non-transformed 103-29 suggests that this strain contains a toxR mutation. The completion of toxR mapping studies currently in progress will reveal whether toxR corresponds to the 35' position reported as the tox-2 locus in P. aeruginosa PA01 (26).

S1 nuclease protection data predicts that the toxR transcript initiates with a uracil residue 20 bp upstream of the toxR translation initiation codon. This distance between the transcript start site and the initiation codon is similar to that found in the phospholipase C gene from P. aeruginosa (24).

Interestingly, sequence analysis of the pGW21::Tn5 has positioned the Tn5 insertion 27 bp upstream of this S1 start site (data not shown). The Tn5 transposon thus appears to be interfering with natural toxR expression by blocking a potential promoter region or other necessary regulatory sequence(s). The presence of this S1 signal along with inverted repeats resembling rho independent transcription terminators downstream of the toxR gene (Fig. 5) suggests that toxR is not part of an operon and is transcribed as a monocistronic message. In addition, this transcript would also contain the 5' GAGCCAUC 3' sequence located in approximately the same position of the toxA leader mRNA. Whether this particular sequence plays any regulatory role in the cell remains to be shown, but may represent a potential 16S rRNA binding region required for translation of these genes.

Very little is known regarding the genetics of ETA regulation in P. aeruginosa. Our preliminary transcriptional studies suggest that the putative toxR gene product may act as a positive regulator of toxA by enhancing transcription of that gene. Trans-acting DNA binding proteins are known to be involved in the regulation of several E. coli genes (27). Thus, it may be possible that the toxR gene product binds to sequences upstream of the toxA gene and promotes efficient transcription. Grant and Vasil (28) have recently reported the finding of two 8 bp direct repeats located upstream of the S1 nuclease mRNA start site for the toxA gene. These sequences could represent potential toxR binding sites.

Taken together, these findings extend our previous observations of toxR (1) and provide the basis for definitive studies regarding the mechanism(s) by which the toxA gene is regulated in P. aeruginosa.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to the following individuals for their helpful discussions and encouragement: Dr. Bruce Holloway, Dr. Randall K. Holmes and Dr. J.N. Reeve. Plasmid pR01614 was kindly provided by Dr. Ronald Olsen. We would also wish to thank Daniel Polonia for his excellent technical assistance. This work was supported by USPHS grant R01 A123429-01 to DRG.

*To whom correspondence and reprint requests should be addressed

REFERENCES

1. Hedstrom, R.C., Funk, C.R., Kaper, J.B., Pavloskis, O.R. and Galloway, D.R. (1986). Infect. Immun. 51, 37-41.

2. Pollack, M., Taylor, N.S. and Callahan, L.T.III (1977). *Infect. Immun.* 15, 776-780.
3. Iglewski, B.H. and Kabat, D. (1975). *Proc. Natl. Acad. Sci. U.S.A.* 72, 2284-2288.
4. Allured, V.S., Collier, R. J., Carroll, S.F. and McKay, D.B. (1986). *Proc. Natl. Acad. Sci. U.S.A.* 83, 1320-1324.
5. Gray, G.L., Smith, D.H., Baldridge, J.S., Harkins, R.N., Vasil, M.L., Chen, E.Y. and Heyneker, H.L. (1984). *Proc. Natl. Acad. Sci. U.S.A.* 81, 2645-2649.
6. Mozola, M.A., Wilson, R.B., Jordan, E.M., Draper, R.K. and Clowes, R.C. (1984). *J. Bacteriol.* 159, 683-687.
7. Bjorn, M.J., Iglewski, B.H., Ives, S., Sadoff, J.C. and Vasil, M.L. (1978). *Infect. Immun.* 19, 785-791.
8. Lory, S. (1986). *J. Bacteriol.* 168:1451-1456.
9. Liu, P.V. (1966). *J. Infect. Dis.* 16, 481-489.
10. Ohman, D.E., Sadoff, J.C. and Iglewski, B.H. (1980). *Infect. Immun.* 28, 899-908.
11. Vieira, J. and Messing, J. (1982). *Gene* 19, 259-268.
12. Boyer, H.W. and Roulland-Dussoix, D. (1969). *J. Mol. Biol.* 41, 459-472.
13. Olsen, R., De Brusscher, G. and McCombie, W.R. (1982). *J. Bacteriol.* 150, 60-69.
14. Iglewski, B.H. and Sadoff, J.C. (1979). *Methods Enzymol.* 60, 780-793.
15. Birnboim, H.C. and Doly, J. (1979). *Nucl. Acids Res.* 7, 1513-1525.
16. Buck, G.A. and Groman, N.B. (1981). *J. Bacteriol.* 148, 153-162.
17. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982). *Molecular cloning. A laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
18. deBruijn, F.J. and Lupski, J.R. (1984). *Gene* 27, 131-149.
19. Maxam, A.M. and Gilbert, W. (1980). *Methods Enzymol.* 65, 499-580.
20. Galloway, D.R., Hedstrom, R.C. and Pavloskis, O.R. (1984). *Infect. Immun.* 44, 262-267.
21. Staden, R. and McLachlan, A.D. (1982). *Nucl. Acids Res.* 10, 141-156.
22. Shepherd, J. (1981). *Proc. Natl. Acad. Sci. U.S.A.* 78, 1596-1600.
23. Hadero, A. and Crawford, I.P. (1986). *Mol. Biol. Evol.* 3, 191-204.
24. Pritchard, A.E. and Vasil, M.L. (1986). *J. Bacteriol.* 167, 291-298.
25. Sastry, P.A., Finlay, B.B., Pasloske, B.L., Paranchych, W., Pearlstone, J.R. and Smillie, L.B. (1985). *J. Bacteriol.* 164, 571-577.
26. Gray, G.L. and Vasil, M.L. (1981). *J. Bacteriol.* 147, 275-281.
27. Pabo, C.O., and Sauer, R.T. (1984). *Ann. Rev. Biochem.* 53, 293-321.
28. Grant, C.R., and Vasil, M.L. (1986). *J. Bacteriol.* 168, 1112-1119.