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Locus of the *Pseudomonas aeruginosa* Toxin A Gene

LARRY F. HANNE, TIMOTHY R. HOWE, AND BARBARA H. IGLEWSKI*

Department of Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201

Received 22 November 1982/Accepted 24 January 1983

The gene for *Pseudomonas aeruginosa* toxin A has been mapped in the late region of the chromosome of strain PAO. Strain PAO-PR1, which produces parental levels of toxin A antigen that is enzymatically inactive and nontoxic, was used as the donor for R68.45 plasmid-mediated genetic exchange. Strain PAO-PR1 (*toxA1*) was mated with toxin A-producing strains, and exconjugates for selected prototrophic markers were tested for the transfer of *toxA1*. The *toxA1* gene was located between *cnu-9001* and *pur-67* at approximately 85 min on the PAO chromosome.

Pseudomonas aeruginosa is primarily an opportunistic pathogen involved with diseases of compromised patients (e.g., cystic fibrosis, neoplasms, burns) (7, 8, 14). *P. aeruginosa* produces several extracellular products which may contribute to its pathogenesis (2, 17, 35). Toxin A, the most toxic of these extracellular products (17), is produced by 80 to 90% of *P. aeruginosa* isolates (3, 23). Suitably activated preparations of toxin A catalyze the transfer of the ADP-ribosyl moiety of NAD^+ onto eucaryotic elongation factor 2, thereby inhibiting protein synthesis (15, 31). Toxin A is associated with virulence of *P. aeruginosa* in some animal models (17, 18, 20, 21, 35) and in some human infections (4, 22).

Our laboratory has recently characterized a mutant strain, PAO-PR1, of *P. aeruginosa* PAO which produces an altered form of toxin A (5). The altered protein is the same size as native toxin A and is immunologically identical to toxin A, yet it is nontoxic (5, 6). The mutation (*toxA1*) in strain PAO-PR1, which does not appear to affect the regulation of toxin A, is undoubtedly located in the toxin A structural gene (5, 6). Similar mutants of corynebacteriophage β proved the bacteriophage location for the structural gene for diphtheria toxin (29, 30). Using strain PAO-PR1 as a donor in conjugal crosses, we have mapped the *P. aeruginosa* toxin A structural gene on the chromosome of strain PAO.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used were derivatives of *P. aeruginosa* PAO and are described in Table 1.

Media and reagents. Vogel-Bonner minimal medium (32) was used for all bacterial crosses involving catabolic markers. Vogel-Bonner minimal medium without citrate was used when anabolic markers were studied. Exconjugants were grown in chelex-treated Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) dialysate containing 50 mM monosodium glutamate and 1% glycerol (1). Trypticase soy broth dialysate was further supplemented with 0.01% adenine (Sigma Chemical Co., St. Louis, Mo.) whenever PAO 944 or PAO 949 was the recipient strain. Antibiotics used were carbenicillin (Geopen; Pfizer Inc., New York, N.Y.) and kanamycin (kanamycin sulfate; Sigma) at 500 μg/ml.

Genetic techniques. Auxotrophs of strain PAO-PR1 (*toxA1*) were isolated after ethyl methane sulfonate mutagenesis (34). The R68.45 plasmid (11) was then transferred from strain PAO 25 *argF leu-10* (R68.45) to the PAO-PR1 auxotrophs so they could be used as donors in the bacterial crosses. R68.45 plasmid-mediated conjugation was performed by simple plate mating (27). Exconjugants which had been purified on selective media were picked into 125 μl of Trypticase soy broth dialysate in microtiter plates (Titertek; Flow Laboratories Inc., McLean, Va.) and grown for 28 h at 32°C. Bacteria were pelleted by centrifugation for 10 min at 900 × *g*. The pellicles were removed by blotting all wells with a sterile 96-well template, and supernatants were then screened for the *toxA1* gene product, which is ADP-ribosyl transferase negative and toxin A antigen positive (5). Supernatant material was assayed for ADP-ribosyl transferase activity as previously described (31).

Enzyme-linked immunosorbent assay. Supernatant material was assayed with an enzyme-linked immunosorbent assay specific for detection of *P. aeruginosa* toxin A antigen. Coating buffer, phosphate-buffered saline (PBS), PBS-Tween, PBS with 1% bovine serum albumin (Sigma), and diethanolamine buffer were as described previously by Voller et al. (33). Briefly, 75 μl of PBS-bovine serum albumin was added to each well of the microelisa plates (Dynatech Laboratories Inc., Alexandria, Va.) previously coated overnight with sheep anti-toxin A (20 μg of protein per ml). Supernatant material (25 μl) from each exconjugant was then transferred to the microtiter wells, bringing the volume to 100 μl. Toxin A antigen was allowed to bind to the antitoxin-coated wells for 2.5 h at 25°C in a humid chamber. Plates were then rinsed four times with PBS-Tween followed by four rinses with PBS. Alkaline phosphatase-conjugated sheep anti-toxin A (100 μl) (16) was added to each well, and the plates

TABLE 1. *P. aeruginosa* strains used

Strain	Relevant genotype[a]	Source
Donor[b]		
PAO-PR1	*toxA1*	Cryz (5)
PAO-PR1, *his*	*toxA1 his*	This paper
PAO-PR1, *arg*	*toxA1 arg*	This paper
Recipients[c]		
PAO 12	*leu-8 pur-136*	Royle (24)
PAO 25	*argF leu-10*	Royle (24)
PAO 236	*met-28 trp-6 lys-12 his-4 proA82 ilvB*	Haas (11)
PAO 944	*thr-9001 cys-54 pur-67*	Royle (24)
PAO 949	*thr-9001 pur-67 cys-59*	Royle (24)
PAO 2249	*catA1 met-9011*	Matsumoto collection[d]
PAO 2368	*met-9020 catA1 nar-9011 cnu-9001 puuE8*	Matsumoto collection[d]

[a] The following abbreviations are used: *arg*, arginine; *cat*, catechol; *cnu*, carnosine utilization; *cys*, cysteine; *his*, histidine; *ilv*, isoleucine-valine; *leu*, leucine; *lys*, lysine; *met*, methionine; *nar*, nitrate reductase; *pro*, proline; *pur*, purine; *puu*, purine utilization; *thr*, threonine; *trp*, tryptophan.

[b] The R68.45 plasmid was transferred to PAO-PR1 auxotrophs. This plasmid confers resistance to carbenicillin, kanamycin, and tetracycline and has chromosome-mobilizing ability (11).

[c] All recipients were toxin A positive.

[d] Obtained from B. Wretlind.

were again incubated for 2.5 h at 25°C. The plates were rinsed as above, 100 μl of the substrate *p*-nitrophenyl phosphate (Sigma) (1 mg/ml in diethanolamine buffer) was added, and the plates were incubated at 37°C for 2 h. The reaction was stopped by adding 50 μl of 60 mM EDTA (Sigma) per well, and the optical density at 405 nm was determined on a Dynatech microelisa reader.

RESULTS

Strain PAO-PR1 (*toxA1*) containing the R68.45 plasmid was mated with toxin A-producing recipient strains, and exconjugates for selected prototrophic markers were tested for the transfer of *toxA1* (Table 2). We observed approximately 25% linkage of *toxA1* to *cnu-9001*, *cys-54*, and *pur-67*. *toxA1* was linked by only 11 and 10%, respectively, to markers earlier (*nar-9011*) and later (*cys-59*) than these three loci. This suggested to us that *toxA1* is located between *cnu-9001* and *pur-67*.

P. aeruginosa PAO 944, PAO 949, and PAO 2368 containing double lesions in the area where the *toxA1* mutation appeared to be located were used to determine which genes surround the *toxA1* locus. Prototrophic recombinants at both *nar-9011* and *cnu-9001* gave 36% coinheritance of *toxA1* as an unselected marker, confirming that *toxA1* is located distal to *cnu-9001*. When cotransfer of both *cys-54* and *pur-67* were selected in strain PAO 944, we observed 92% coinheritance of *toxA1*. Since prototrophic recombinants for the next set of markers (*pur-67* and *cys-59*) gave only 10% coinheritance of *toxA1*, the *toxA1* gene is clearly between *cnu-9001* and *pur-67*.

The R68.45 plasmid is capable of mobilizing at least 10 min of chromosomal material (11). Since only approximately 25% linkage of *toxA1* was obtained with the closest markers (*cnu-9001* and *pur-67*) by R68.45 plasmid-mediated exchange, the *toxA1* gene is not physically immediately adjacent to either of these markers. If we assume nonpolar chromosome transfer in all these crosses and note that *toxA1* linkage to *cnu-9001* and *pur-67* individually is 26 and 25%, respectively, the *toxA1* gene must be located between these two genes at approximately 85 min on the PAO chromosome. Since cotransduction of any two genes requires that they be within 1 to 2 min of each other, transductional analysis was not performed.

DISCUSSION

Genetic analysis of virulence factors from bacteria is simplified in many cases since these

TABLE 2. Linkage of *toxA1* to selected markers[a]

Selected marker(s)	PAO chromosome location (min)[b]	Linkage (%)[c]
ilvB	8	0/10 (0)
lys-12	20	0/10 (0)
pur-136	25	0/29 (0)
proA82	40	0/30 (0)
argF	55	0/20 (0)
catA1	65	0/20 (0)
nar-9011	75	5/45 (11)
nar-9011–cnu-9001[d]	75–80	12/33 (36)
cys-54	80	33/118 (28)
cnu-9001	80	21/80 (26)
cys-54–pur-67	80–90	22/24 (92)
pur-67	90	28/113 (25)
pur-67–cys-59	90–95	2/20 (10)
cys-59	95	9/86 (10)

[a] Recipient PAO stains with characterized auxotrophic lesions were converted to prototrophy by R68.45 plasmid-mediated transfer from strain PAO-PR1 (*toxA1*). Prototrophic exconjugants for selected markers were screened for coinheritance of *toxA1* as an unselected marker.

[b] Locations of late markers from the FP2 plasmid origin of transfer are approximate.

[c] Linkage of *toxA1* to markers later than 65 min represents values from three independent crosses.

[d] Exconjugants which were selected for prototrophy at *cnu-9001* and found to be prototrophic at *nar-9011* were then tested for *toxA1* transfer.

factors are often coded for by extrachromosomal elements (9, 19, 25, 29, 30). We are unaware of any chromosomally coded exotoxins for which the structural gene location is known. For the performance of such an analysis, a system for genetic exchange must exist, and a strain must be derived with a lesion in the desired structural gene.

Genetic analysis in *P. aeruginosa* has until recently been limited to transduction and FP2 plasmid-mediated genetic exchange (12). A variant of the R68 plasmid, R68.45, has been described which has chromosome-mobilizing ability from multiple random sites around the chromosome (11). This plasmid can mobilize 10 min of donor chromosome and has facilitated genetic analysis of markers in the late region of the chromosome (13, 28). We used the R68.45 plasmid to mediate transfer of chromosome material from strain PAO-PR1 (*toxA1*) and selected prototrophic recombinants for characterized auxotrophic lesions in recipient strains. Selection of prototrophic recombinants for two markers convincingly demonstrated the location of the *toxA1* gene to be between *cnu-9001* and *pur-67*.

Gray and Vasil (10) have mapped two loci (*tox-1*, *tox-2*), which appear to regulate expression of *P. aeruginosa* toxin A, at 36 to 39 min on the PAO chromosome. One of their toxin A-deficient mutants, PAOT10 (*tox-1*), is pleiotropic and may involve a defect in secretion of extracellular proteins, similar to the *xcp* mutants described previously by Wretlind and Pavlovskis (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, D62, p. 48). The other mutant, PAOT20 (*tox-2*), appears to have a lesion specific to toxin A production (10). It would be tempting to speculate that the *tox-2* locus is adjacent to or near the toxin A structural gene. Our data show that this is not the case. Rather, the gene coding for the toxin A structural gene (*toxA1*) is located at 85 min, quite some distance from the *tox-2* locus. This suggests that the *tox-2* locus may code for a derepressor which acts in a *trans* mode to activate transcription of the toxin A structural gene. Alternatively, the *tox-2* locus may code for a product specifically involved in processing or secretion of toxin A. It would be of interest to see whether any of the specific toxin A iron-deregulated mutants (26) map near the *tox-2* locus or the *toxA1* locus.

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