PRODUCTION OF EXOTOXINS WITH ADP-RIBOSYL TRANSFERASE ACTIVITY BY *PSEUDOMONAS AERUGINOSA*

by

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I. Introduction and Statement of the Problem

Pseudomonas aeruginosa is an opportunistic pathogen which causes serious and sometimes fatal infections in the compromised host. The organism is capable of producing a wide variety of extracellular products which may contribute to its pathogenicity (61, 62). Among these is an extracellular protein, toxin A, discovered by Liu (59). Toxin A is cytotoxic and lethal to many cells and animals (3, 59, 70). The mechanism of action of toxin A is identical to diphtheria toxin fragment A (11, 45-47). Both toxins inhibit eukaryotic protein synthesis by catalyzing the transfer of the adenosine diphosphate ribose (ADPR) moiety of nicotinamide adenine dinucleotide (NAD) onto elongation factor 2 (EF-2), rendering the ADPR-EF-2 inactive in protein synthesis (11, 46, 47). While the mechanism of action of toxin A is well understood, its role in disease remains to be clarified. Furthermore, toxin A is not formed constitutively by A toxinogenic strains of *P. aeruginosa*. The factors regulating its production have not been clarified.

The purpose of this study is to elucidate *in vitro* and *in vivo* production of toxins produced by *P. aeruginosa* which have ADP-ribosyl transferase activity. The specific aims of this research are:

i. Establish the incidence of toxin A production among clinical isolates and laboratory strains of *P. aeruginosa*.

ii. Examine the factors that regulate yields of *Pseudomonas* toxins *in vitro*.

iii. Investigate the possible existence of *P. aeruginosa* ADP-ribosyl transferases distinct from toxin A.

iv. Demonstrate the *in vivo* production of *P. aeruginosa* exoenzyme S in an appropriate animal model.

Increased understanding of the *in vitro* and *in vivo* production of ADP-ribosyl transferases from *P. aeruginosa* may provide clues relating to the pathogenesis, prophylaxis, and therapy of *P. aeruginosa* infections.

II. Literature Review

A. Morphology, Physiology and Genetics of *P. aeruginosa*

The cells of *Pseudomonas* are typically straight rods measuring 0.5-1.0 μm by 1.5-4.0 μm. *P. aeruginosa* is motile due to the presence of a single polar flagella and the organism also possesses pili or fimbrae which are found as a polar tuft (79, 94). *Pseudomonas* is known for its pigmentation and several types of pigments are produced. The most common are pyocyanine (a phenazine pigment), fluorescein, and a melanin-like brown pigment termed pyomelanin (61, 62 79). *Pseudomonas* possesses a diffuse slime polysaccharide coat which differs from the hard coat (capsule) surrounding many other bacteria (61, 62). The slime layer is believed to be antiphagocytic (94).

The wall and membrane structure of *P. aeruginosa* grossly resembles those found on most other gram-negative bacteria; an outer membrane and an inner membrane sandwich a peptidoglycan layer between them. The other major component of the cell wall is the lipopolysaccharide (LPS). The LPS from *Pseudomonas* has many physical and chemical properties in common with the LPS from members of the *Enterobacteriaceae*. However, important differences do exist as is evident from the low (endo-) toxicity of *P. aeruginosa* LPS (16, 41, 42, 56, 69).

P. aeruginosa is most often referred to as an aerobe in relation to oxygen, but it can utilize nitrates as a terminal electron acceptor in the absence of oxygen (8). It has a much wider growth temperature range than most pathogens being able to grow equally well

at 20° C and 37° C (62). P. aeruginosa catabolizes glucose and other hexoses by utilizing the Entner-Douderoff pathway instead of the Embden-Myerhoff pathway (9) and it has a functional tricarboxylic acid cycle and electron transport system (8). Pseudomonas has enormous metabolic potential. The organism can grow on more than 80 different organic compounds as the sole source of carbon including carbohydrates, alcohols, saturated and unsaturated fatty acids, amino acids, amines and amides (79). In addition, it has remained viable and even grown in distilled water (19) and in dilute solutions of antiseptics and disinfectants (19).

The genome of P. aeruginosa consists of a chromosome (2-3 x 10^9 daltons) (39) and one or more plasmids of 10-312 x 10^6 daltons (33, 39). Both the chromosome and the plasmids are attached to the bacterial cytoplasmic membrane. The plasmids are of several types (38, 39) including sex factors (FP; fertility plasmids) that mobilize the bacterial chromosome during conjugation, antibiotic resistance plasmids (RP; resistance plasmids), degradative plasmids and cryptic plasmids. All three types of gene transfer (conjugation, transformation and transduction) take place in Pseudomonas. Of the three, conjugation and transduction have been used extensively to study the genetics and the chromosomal map of P. aeruginosa (38, 39).

Epidemiologically, there are a number of ways in which P. aeruginosa strains are typed. These include serotyping, pyocin typing and phage typing. A variety of serotyping schemes have been

developed. Unfortunately, no single scheme has enjoyed widespread approval and three or four different schemata are commonly used, including those developed by Habs (32), Verder and Evans (113) and Fisher et al. (24). Generally these methods are based on serotyping by use of antisera directed at the somatic "O" LPS antigens.

Bacteriocins are antibiotic substances produced by many bacteria, and pyocins are the bacteriocins produced by Pseudomonas (20, 21, 40). An organism that produces a pyocin is resistant to that pyocin. Other Pseudomonas strains will either be susceptible or resistant depending on the presence or absence of receptors for that pyocin on their cell wall. Farmer and Herman (21) have developed a pyocin typing scheme involving 18 indicator strains of P. aeruginosa. The organism to be typed is grown in broth and pyocin production is induced by the addition of mitomycin C. The pyocin extract is then spotted on a lawn of each of 18 indicator strains, and the plates are later scored for the inhibition of growth at the area on the plate where the pyocin extract was spotted. Most workers in the field agree that the initial step in Pseudomonas typing should be a serological test. Often times, however, it is necessary to also do pyocin typing in order to determine the relatedness of two strains with identical serotypes. Phage typing of P. aeruginosa is still in the experimental stages and is not routinely used at this time (19).

P. aeruginosa is noted for its resistance to most of the commonly used antibiotics. There are two major mechanisms for this resistance; selective exclusion properties of the

outer membrane and resistance plasmids. The outer envelope layers of P. aeruginosa, in common with other gram-negative bacteria, restrict the entry of a number of antibiotics (68). Plasmids conferring drug resistance in P. aeruginosa were first identified by Lowbury and his collaborators in England during the late 1960's (64). Now a variety of R-factors have been identified worldwide in Pseudomonas. These differ in antibiotic resistance patterns and in molecular size (39). Resistance to the antibiotics streptomycin, carbenicillin, kanamycin, neomycin, tetracycline, sulphonamide, ampicillin and gentamicin are most commonly coded for by R-plasmids (39). However, any given plasmid usually carries genes for resistance to only two or three antibiotics (39). It has been shown that R-plasmids are mutually transferrable between P. aeruginosa and a wide variety of other gram-negative genera (38, 78).

B. Disease Entities Caused by P. aeruginosa

P. aeruginosa is regarded as a saprophyte in that it is a natural inhabitant of soil and fresh water (79). It is often a cause of infection in man and other animals, especially in the compromised host. P. aeruginosa is a particularly severe problem in hospitalized patients partly because of its ability to persist in environments where other common pathogens are killed. Thus, it is often found in moist environments such as sinks, baths, whirlpools, and inhalation equipment (19, 37). P. aeruginosa has been found in high numbers in uncooked vegetables comprising salads that are fed to hospitalized patients (19, 51) and in ornamental plants and

flowers that are often found in hospital rooms and wards (51). Pseudomonas thus differs from the bacteria causing the classical bacterial diseases (diphtheria, cholera, etc.) in being found in high numbers in the hospital environment, thus complicating its control. The situation becomes even more ominous when one is aware that between 3% and 25% of healthy individuals are gastrointestinal carriers of P. aeruginosa (19).

Healthy humans are usually not infected by P. aeruginosa, a notable exception being chronic ear infections (19). On the other hand, compromised and immunosuppressed patients run a high risk of P. aeruginosa infections. In particular, cystic fibrosis, burn, and leukemia patients are most susceptible. One of the hallmarks of P. aeruginosa is its great propensity to invade vascular walls, often leading to the characteristic skin lesion known as erythyma gangrenosum (114). Pseudomonas septicemia is particularly severe with a mortality rate of from 60-90% despite the use of the best available antibiotics and supportive care (25). This mortality rate far exceeds the rate seen from bacteremia with other gram-negative organisms affecting immunosuppressed patients (25), and implies that P. aeruginosa possesses special virulence factors not shared by members of the Enterobacteriaceae.

Infections caused by P. aeruginosa are a grave threat to patients with extensive burns. The organism can readily colonize the burn wound and often times may be spread hematogenously and cause systemic sepsis and Pseudomonas pneumonia, conditions with a poor prognosis (25, 90, 114). Another group of patients that are

especially susceptible to P. aeruginosa infections are those suffering from neoplasia, particularly acute leukemias (101, 106, 114, 119). The critical role of granulocytopenia in these patients is shown by the increase in Pseudomonas septicemia in patients with white blood cell counts below 1,000 per ml of blood (106, 114).

Cystic fibrosis (CF) is the most common lethal inherited (autosomal recessive) disease in Caucasians with an incidence of 1 in 2,000 live births (14, 114). More than 50% of patients with CF die before reaching the age of 21 (14). The biochemical or physiological lesions of the disease are not fully understood (14). However, chronic obstructive pulmonary disease is the most serious aspect of CF (14, 114). Pulmonary difficulties arise from an inadequate removal of mucous from the bronchial tree and also from poor ciliary movement (14, 114). P. aeruginosa is the organism most frequently colonizing the respiratory tract of CF patients (115). An interesting finding is that 50-70% of P. aeruginosa strains isolated from CF patients are mucoid in colonial morphology (15, 31, 114), possessing a viscous extracellular polysaccharide that surrounds each bacterial colony. Only rarely are P. aeruginosa strains from other clinical sources found to be of the mucoid type. While the viscous nature of the polysaccharide may contribute to the respiratory problems seen in chronically infected CF patients, its precise role remains unclear (31, 114). Govan has shown that mucoid P. aeruginosa strains are more resistant to carbenicillin than non-mucoid strains (31), and this may partly explain the prevalence of mucoid strains

in the lungs of CF patients undergoing long term antibiotic therapy.

P. aeruginosa has become one of the most common causes of severe corneal infection in man (13). In keeping with its role as an opportunistic pathogen, P. aeruginosa does not normally infect the intact corneal epithelium (44). The cornea must be previously damaged (by a scratch, for example) for a Pseudomonas infection to occur. The keratitis produced by P. aeruginosa is one of the most rapidly spreading bacterial diseases of the cornea and is very destructive, often leading to permanent blindness (13, 44).

The treatment of patients with P. aeruginosa infections varies depending on the site of infection and the fitness of the host. As mentioned earlier, antibiotic therapy of P. aeruginosa infections is complicated by the fact that the organism is resistant (and can acquire resistance) to a variety of the commonly used antibiotics. In general, the major antibiotics employed to treat P. aeruginosa infections are gentamicin (or tobramycin) and carbenicillin (or ticarcillin) (114). Cystic fibrosis patients with P. aeruginosa upper respiratory infections are sometimes administered aerosolized antibiotics (neomycin, colistin or carbenicillin) in an attempt to deliver the antibiotic directly to the lungs (114). In addition, mucolytic agents such as N-acetylcysteine and bronchodialators have been administered to CF patients in an attempt to clear the air passages (114). Treatment of superficial Pseudomonas infections include topical application of dilute acetic acid, mafenide acetate (sulfamylon), silver nitrate, and gentamicin cream (114). In burn patients where the initial therapeutic emphasis is on the

prevention of establishment of Pseudomonas at the burn site, silver sulfadiazine (114) has been highly effective. A drawback is that the use of silver sulfadiazine has been associated with the emergence of other potentially life-threatening organisms such as fungi and Providencia stuartii (114). Due to the ability of P. aeruginosa to acquire resistance to antibiotics the treatment of Pseudomonas infections is ever-changing and modifications in the therapeutic regimen often occur in a relatively short period of time.

Other therapeutic measures such as granulocyte transfusions and passive immunization with immune gamma globulin have looked promising in experimental animal models (12, 17, 34, 35, 49) but have done little to decrease the morbidity or mortality in human infections (114).

C. Virulence Factors of P. aeruginosa

P. aeruginosa produces a wide variety of extracellular products that may contribute to its pathogenicity (61, 62). However, the exact role that they play in disease is only beginning to be understood. Even though P. aeruginosa is a gram-negative bacterium (that by definition must possess LPS [endotoxin]) the LPS isolated from the cell wall of P. aeruginosa is not nearly as toxic as the LPS isolated from the Enterobacteriaceae (16, 41, 42, 56, 61, 69). A number of extracellular products produced by P. aeruginosa also have inherent low toxicities or are produced at such low levels that they are not important in diseases. These include a phytotoxic factor (61), pigments (61), and hydrocyanic acid (61, 62).

Based on studies using animal models and isolated cells and tissues, a number of extracellular substances produced by P. aeruginosa appear to have the potential to contribute to its pathogenicity. Among these are surface slime, phospholipase, hemolysins, leukocidin, enterotoxin, Z toxin, proteases (elastase), and exotoxin A. These will be discussed in the following pages. The roles of other potential virulence factors such as pili and flagella are presently poorly studied and will not be discussed in this review.

1. Surface slime. Instead of the hard polysaccharide capsule that coats many other gram-negative bacteria, P. aeruginosa has a loosely defined slime polysaccharide layer (61, 62). The data are conflicting on the toxicity of purified slime. Alms and Bass (1, 2) reported that doses as high as 90 µg were not toxic to mice. In contrast, Sensakovic and Bartell (102) reported that the response of mice to intraperitoneal (i.p.) injection of purified slime was leukopenia and death. It should be noted that the dose administered in this later study (102) was 200 µg and that contamination of the slime preparation with other virulence factors of P. aeruginosa was not excluded. Although definite evidence does not exist, Sadoff (94) speculates that the surface slime may contribute to the pathogenicity of P. aeruginosa by being anti-phagocytic.

2. Hemolysins. P. aeruginosa produces at least two substances with hemolytic activity; a heat-stable glycolipid and a heat-labile phospholipase C (lecithinase). The chemical structure of the glycolipid was elucidated by Jarvis and Johnson (48) as

consisting of two moles of L-rhamnose and two moles of β-hydroxy-decanoic acid. The purified glycolipid was relatively non-toxic, taking 5 mg to kill a mouse (48). Although Johnson and Jarvis did not examine the hemolytic properties of the glycolipid, the identity of the glycolipid with hemolytic activity was shown by Sierra (103).

Phospholipase C is a lecithinase which liberates phosphorylcholine from lecithin (18, 61, 62). To date there is no evidence that the phospholipase C plays a role in the virulence of P. aeruginosa (61, 62). Liu speculates that the presence of a lecithinase in the lungs of P. aeruginosa pneumonia patients has the potential to be a virulence factor, since the normal surfactant lining the lungs and preventing atelectasis is lecithin (62).

3. Leukocidin. Scharmann has characterized a substance produced by several strains of P. aeruginosa that is cytotoxic to leukocytes from various animal species (96-100). The substance is not cytocidal to erythrocytes or thrombocytes. This leukocidin is a cell-bound protein of molecular weight 27,000 daltons (99, 100). The cytotoxic action of leukocidin on bovine leukocytes is characterized by an increased permeability of the plasma membrane for low molecular weight markers (96). There is no evidence at the present time that leukocidin plays a role in disease, although a purified preparation of leukocidin has a minimal lethal dose (LD_{50}) of 1 μg in mice (99).

4. Z toxin. Ludovici (65) reported the presence of a toxic material produced by P. aeruginosa growing in HEp-2 tissue culture

cells. This material, Z toxin, caused the formation of virus-like plaques in the tissue culture monolayer (65, 66). The toxin has a molecular weight of about 70,000 daltons. Unfortunately, cross-neutralization studies with specific toxin A antiserum or antisera directed at other virulence factors of P. aeruginosa have not been reported in the literature. However, Ludovici reported that Z toxin was more heat-stable than exotoxin A (66). The toxicity of purified toxin Z for whole animals has not been determined and the role, if any, of this substance in the pathogenesis of P. aeruginosa infections has not been elucidated.

5. Enterotoxin. P. aeruginosa is associated with diarrheal conditions (61). Often known as five-day fever or Shanghai fever, these diarrheas present with dysentery-like symptoms (62). Kubota and Liu (53) demonstated the presence of an enterotoxin from P. aeruginosa cultural supernatants that caused fluid accumulation in ligated loops of rabbit ileums. This test is routinely used to detect the presence of other bacterial enterotoxins (22). The crude substance appeared to be protein in nature because it was heat-labile and readily destroyed by proteolytic enzymes. It should be noted that rabbits similarly injected with purified proteases from P. aeruginosa also showed fluid accumulation in their ileal loops (22). Thus the enterotoxin described by Kubota and Liu may be Pseudomonas proteases. In addition, a vascular permeability factor produced by some strains of P. aeruginosa has been described (54). Its relationship to the enterotoxin or proteases is not known.

6. Proteases. Of all the known potential virulence factors of P. aeruginosa, exotoxin A and the proteases are the most toxic (61, 62). Kreger et al. (52), Wretlind et al. (115, 117), and Morihara and Tsuzuki (72) have shown that supernatants of P. aeruginosa contain at least three distinct proteases. Wretlind refers to these as protease I, II, and III with isoelectric points of 8.5, 6.6 and 4.5 respectively (115, 117). He reports that all three proteases have molecular weights in the 19,000 to 25,000 dalton range, although Morihara reports higher molecular weights (72) which may represent dimers of the molecules reported by Wretlind. Protease II accounts for greater than 75% of the total proteolytic activity of crude supernatants (115, 117). In addition, protease II has elastolytic activity and is referred to as an elastase (52, 72, 115, 117). Mull and Callahan (77) have shown that 86% of P. aeruginosa strains isolated from human infections produced elastolytic activity, and Morihara and Tsuzuki (72) and Wretlind et al. (116) have shown that a similar percentage of P. aeruginosa clinical isolates produced elastolytic and proteolytic activity. Thus, the majority of P. aeruginosa strains isolated from human infection have the ability to produce proteases and an elastase. The determination of the ability of P. aeruginosa strains to produce proteases in vivo is complicated by the presence of endogenous proteases in the cells, tissues and fluids of animals.

Most evidence leads one to believe that proteases contribute significantly to the pathogenesis of P. aeruginosa corneal

infections (4, 23, 52). However, the role of proteases in the pathogenesis of other types of Pseudomonas infections is not well understood. On a weight basis, proteases are much less toxic to mice and other experimental animals than exotoxin A (61). The minimum lethal dose (LD_{50}) for a mouse is 75-100 µg of purified Pseudomonas proteases (61) compared to 60-200 ng of purified toxin A (6, 46, 57).

Recently, Snell and co-workers (104) have presented evidence suggesting that both proteases and exotoxin A produced by P. aeruginosa multiplying in situ in a burned mouse are virulence factors. However, a very small number of strains were tested (104) and the strains were not isogenic. Wretlind (115, 118) attempted to produce isogenic protease negative ($prot^-$) mutants from a $prot^+$ parent using P. aeruginosa strain PAK. He was unsuccessful in isolating true $prot^-$ mutants, although he did isolate hypoproteolytic mutants that produced 1-25% of the parent protease levels (115, 118). The hypoproteolytic mutants were not completely isogenic to the parent as the production of other extracellular products were also altered. Wretlind tested the virulence of his mutants in three separate mouse models and found no detectable difference in virulence between the hypoproteolytic mutants and their parent. Further studies using true $prot^-$ isogenic mutants and other animal models are necessary before the precise roles that proteases play in disease are known. It will also be necessary to elucidate the relative contributions of the three individual proteases in P. aeruginosa infections.

7. Exotoxin A. On a weight basis, toxin A is by far the most toxic substance produced by P. aeruginosa (61, 62). The structure-function relationships, enzymatic properties and the in vitro and in vivo toxicities of toxin A will be discussed in detail in the next section.

D. Mechanism of Action of Toxin A

Purified A toxin is a heat labile protein with a molecular weight of approximately 70,000 daltons (45, 46, 57, 107, 111). As little as 60 ng of toxin A is lethal for mice (6). Correspondingly small amounts of toxin A are lethal for rabbits, dogs, and rhesus monkeys (3, 83), produces a shock syndrome in dogs and monkeys distinct from that produced by endotoxin (3), and results in death of epithelial, endothelial and stromal cells followed by necrosis when injected into rabbit corneas (44).

Exotoxin A has been purified in a number of laboratories using a variety of classical biochemical techniques (6, 46, 59, 63, 112) and more recently by affinity chromatography using insolubilized anti-A immunoglobulin (107). Toxin A inhibits protein synthesis in livers, kidneys, and spleens of intoxicated mice (87), in cultured mammalian cells (83, 84) and in cell-free systems derived from eukaryotic cells (45-47). The inhibition of protein synthesis by toxin A requires nicotinamide adenine dinucleotide (NAD) and results in inactivation of eukaryotic elongation factor 2 (EF-2) in a manner identical to diphtheria toxin fragment A (11, 45-47, 80).

Unmodified EF-2 is required for the translocation of the nascent polypeptide chain from the A site to the P site on the eukaryotic ribosome (11, 80). The ADPR-EF-2 complex is inactive in its translocase function (11, 45-47, 80). The reaction catalyzed by toxin A can be reversed by fragment A of diphtheria toxin producing active EF-2 and vice versa (47). In addition, Chung and Collier (10) recently showed that enzymatically active peptides of Pseudomonas toxin A (M.W. 27,000 daltons) and diphtheria toxin (M.W. 23,000 daltons) have virtually identical specific activities in both the ADPR-transferase and NAD glycohydrolase reactions, comparable Michaelis constants for NAD and EF-2 and similar inhibition constants (Ki's) for adenine and nicotinamide containing analogues of NAD. These results indicate that these two toxins have identical enzymatic activities and it is probable that the catalytic centers contain the same or similar amino acids.

Toxin A is synthesized as a single polypeptide chain (10, 58, 111) with an isoelectric point of 5.1 and it contains 4 disulfide bridges (57). Toxin A is produced by P. aeruginosa as a toxic proenzyme (10, 58, 111) and is virtually if not entirely devoid of enzymatic activity (10, 57, 58, 111). The ADPR-transferase activity is expressed when the molecule is denatured and reduced (57, 58, 111) or when it is cleaved by Pseudomonas proteases to yield an enzymatically active 27,000 dalton fragment (10,111). Thus fragmentation such as is absolutely required for expression of diphtheria toxin enzymatic activity (11, 80) is not absolutely required but can occur with Pseudomonas toxin A (10, 57, 58, 111). The enzymatically

enzymatically active 27,000 dalton fragment are diminished or lacking in toxicity for mouse L-cells and mice (10, 111). Thus, enzymatic activity alone is not sufficient for the expression of toxicity by toxin A (58, 111). In this regard toxin A is similar to diphtheria toxin, Vibrio cholerae and Escherichia coli (labile toxin) enterotoxins as well as the plant toxins abrin and ricin. Each of these toxins has been shown to consist of two separable components both of which are necessary for the expression of toxicity (11, 27, 28, 80, 91). There is an effector portion or structural form of these toxins usually designated A (A for active) which catalyzes a specific intracellular reaction and a carrier component termed B (B for binding) which is responsible for making initial contact with the surface of susceptible cells. While the active (A) fragment of toxin A has been isolated and characterized (10, 111) the binding fragment (B) has only tentatively been identified (10, 111). Furthermore, it is clear that fragmentation such as is absolutely required for expression of diphtheria toxin enzymatic activity (11, 80) is not absolutely required but can occur with Pseudomonas toxin A (10, 57, 58, 111).

E. Cell Specificity and Immunological Cross-reactivity of Toxin A and Diphtheria Toxin

Despite their identical enzymatic activities, P. aeruginosa toxin A and diphtheria toxin affect different lines of cultured cells in a distinctive manner (70, 71, 110). Thus HeLa cells are more sensitive to diphtheria toxin than to toxin A, whereas mouse L cells are very sensitive to toxin A but highly resistant to diphtheria toxin

active peptide of toxin A contains no sulfhydryl groups (10) and its appearance does not require treatment with reducing agents (10, 111). The reduced and denatured but enzymatically active toxin or the enzymatically active 27,000 dalton fragment are diminished or lacking in toxicity for mouse L-cells and mice (10, 111). Potentiation of enzymatic activity is accompanied by a loss in toxicity (58, 111). Thus, enzymatic activity alone is not sufficient for the expression of toxicity by toxin A (58, 111). In this regard toxin A is similar to diphtheria toxin, Vibrio cholerae and Escherichia coli (labile toxin) enterotoxins as well as the plant toxins abrin and ricin. Each of these toxins has been shown to consist of two seperable components both of which are necessary for the expression of toxicity (11, 27, 28, 80, 91). There is an effector portion or structural from of these toxins usually designated A (A for active) which catalyzes a specific intracellular reaction and a carrier component termed B (B for binding) which is responsible for making initial contact with the surface of susceptible cells. While the active (A) fragment of toxin A has been isolated and characterized (10, 111) the binding fragment (B) has only tentatively been identified (10, 111).

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(70, 110). Secondary cultures of chick embryo fibroblasts appear to be equally sensitive to both toxins (70, 110). Thus it appears that toxin A and diphtheria toxin differ with respect to their initial interactions with cells; either the cell receptors with which these toxins interact are different and/or entry and activation of these two toxins are different.

It is known that fragment B of diphtheria toxin will compete with and in sufficient quantity prevent subsequent intoxication of susceptible cells by diphtheria toxin (120). Vasil and Iglewski (110) have recently found that the addition of diphtheria toxin fragment B (at 100X the concentration of diphtheria toxin) will prevent intoxication of chick embryo fibroblasts with diphtheria toxin; however, diphtheria toxin fragment B will not prevent intoxication of chick embryo fibroblasts with P. aeruginosa toxin A even when added at 300X the concentration of A toxin (110). While not ruling out that other differences exist, these observations suggest that these two toxins interact with different cell receptors. Furthermore, antiserum against P. aeruginosa toxin A does not neutralize diphtheria toxin fragment A (46) and antibody against diphtheria toxoid (57) or diphtheria toxin fragment A (46) fail to neutralize A toxin. Since most of the neutralizing antibodies produced in response to diphtheria toxoid are directed against its B fragment (82, 92), these observations further suggest that the binding portions (B fragments) of these two toxins are distinct. Published work from Dr. Iglewski's laboratory (46) and others (10, 57, 70) have failed to detect any

immunological cross-reactivity between diphtheria toxin and its A fragment and P. aeruginosa toxin A as measured by neutralization of enzymatic activity, immunodiffusion analysis, or neutralization of tissue culture toxicity. However, in light of the similarities between the catalytic centers of diphtheria toxin and toxin A it would not be surprising if more sensitive techniques would detect immunological cross-reactivity between the two toxins.

F. Toxin A in vivo

Although it is evident that exotoxin A causes the NAD dependent inactivation of EF-2 in vitro (45-47), it was necessary to demonstrate that the same relationship was occurring in vivo. Iglewski et al. (47) have shown that mice injected with μg quantities of exotoxin A have a decrease in functional EF-2 levels in several organs, including the liver, heart, kidney, spleen, and lungs. Inhibition was greatest in the liver, where over 90% of the EF-2 was inactive (47). Further studies by Pavlovskis et al. (85, 86) have utilized the burned mouse model developed by Stieritz and Holder (105). In these experiments, infection with an exotoxin A producing strain (PA-103) and a non-producing strain (WR-5) were compared. Organs from mice infected with the toxinogenic PA-103 contained considerably less EF-2 activity than did organs from uninfected controls. In contrast, tissue active EF-2 levels from burned mice infected with the non-toxinogenic WR-5 were not markedly reduced below those derived from uninfected controls (85). Passive immunization of the mice with antitoxin (A) prior to infection with the PA-103 strain

prevented the subsequent inactivation of EF-2 (85, 86) and was also found to enhance the survival of burned mice that had been infected with the toxin A producing strain (PA-103) of P. aeruginosa (86). The same passive immunization of burned mice infected with the non-toxinogenic strain (WR-5) did not enhance the survival (86). These results suggest that P. aeruginosa toxin A is produced in vivo and that it inactivates EF-2 in vivo. In addition, these results and others (95, 104) show that exotoxin A contributes to the mortality of experimental animals infected with toxinogenic strains of P. aeruginosa.

Recently, Snell and coworkers have presented evidence which suggests that both proteases and exotoxin A produced by P. aeruginosa multiplying in situ in a burned mouse model are virulence factors (104). When challenge with PA-103 was supplemented by injection with 10 µg of purified Pseudomonas protease, the LD_{50} was reduced by a factor of 1,000 and the mean time to death was significantly shortened (104). Their data suggest that both exotoxin A and proteases are required for the full expression of virulence in P. aeruginosa infections.

There is as yet no direct evidence that exotoxin A is produced in vivo in humans infected with toxinogenic P. aeruginosa isolates. However, Pollack et al. (89) detected the presence of antibody to exotoxin A in human sera from patients infected with P. aeruginosa using a cytotoxicity-neutralization assay. Saelinger et al. (95) have presented evidence for exotoxin A production during

experimental Pseudomonas infections of burned mice. ADPR-transferase activity was found in saline extracts of burned infected skin but not in similar extracts of burned uninfected control mice (95). In addition, evidence was presented that toxin A was detectable in the sera of some mice infected with a toxinogenic strain (M-2) of P. aeruginosa. This report (95) presented the first direct evidence that toxin A is produced in vivo by toxinogenic strains of P. aeruginosa. Although similar evidence is lacking for the in vivo production of toxin A in humans infected with P. aeruginosa, this might be expected in light of the evidence of Saelinger et al. (95) and Pollack et al. (89) discussed above.

G. Physiology of Toxin A Production

Liu reported that a number of factors influenced the production of toxin A by P. aeruginosa strain PA-103 (60). Thus aeration of the culture, an incubation temperature of approximately 32° C, and the presence of glycerol as a carbon source appeared to maximize the production of high yields of toxin A. In addition, a non-dialysable component of trypticase soy broth was found that enhanced the growth of the organism but inhibited toxin A production (60). The non-dialysable inhibitor was identified as nucleic acid (probably RNA) (60). Thus toxin A is not produced constitutively by toxinogenic strains of P. aeruginosa and it is likely that specific regulatory systems control the synthesis and secretion of toxin A in a manner that is distinct from the regulation of bulk protein synthesis. Liu has formulated a medium that is suitable for the

production of high yields of toxin A by strain PA-103 (60). It consists of the dialysate from trypticase soy broth, 0.05 M monosodium glutamate and 1% glycerol (60).

It has long been known that the production of diphtheria toxin is regulated by the concentration of iron in the culture medium (11, 80, 81). Reports also indicate that the production of Shigella dysenteriae toxin is regulated by iron (109). In both cases, the final yield of toxin is inversely proportional to the amount of iron present in the culture medium (this relationship holds only at low iron concentrations). The effect of iron on yields of toxin A produced by toxinogenic strains of P. aeruginosa has not been reported in the literature, but will be investigated in this thesis research. It is interesting that the yields of the P. aeruginosa pigments pyocyanine and fluorescein (5, 26, 50, 108) are under the control of iron, being produced in maximum yields in media containing low amounts of iron. The effect of iron on the yields of other extracellular products of P. aeruginosa has not been reported in the literature.

H. Other ADPR-transferases

To date seven prokaryotic proteins have been identified as ADPR-transferases. These are diphtheria toxin, P. aeruginosa exotoxin A, cholera toxin (73, 75), a N4 phage enzyme (88), two T4 phage enzymes (30, 36, 67) and E. coli labile-toxin (74). All of the prokaryotic ADPR-transferases are mono-ADPR-transferases, transferring only monomers of ADPR to the acceptor proteins (36). The acceptor protein for diphtheria toxin and exotoxin A is EF-2 as has been

described in detail in previous pages. Cholera toxin exerts its effects on the small intestine (and a variety of other cells and tissues) through the activation of adenylate cyclase (22, 27). Cholera toxin is thought to cause the ADP-ribosylation of the regulatory GTPase (MW 42,000) of the adenylate cyclase system (7, 29). Since the conversion of the adenylate cyclase from an active to an inactive state requires the removal of GTP from the regulatory site of the adenylate cyclase (7, 29), it is easy to imagine the manner in which cholera toxin, by ADP-ribosylating the regulatory GTPase, activates adenylate cyclase. It is interesting that diphtheria toxin, Pseudomonas toxin A and cholera toxin are analogous enzymes in that they ADP-ribosylate GTP binding proteins (EF-2 and GTPase).

E. coli labile toxin is responsible for symptoms that mimic clinical cholera (22, 28). The toxin has been shown to activate adenylate cyclase (27, 28). Recently, E. coli labile-toxin has also been shown to possess both NAD glycohydrolase activity and ADPR-transferase activity (74). Although the substrate for the ADP-ribosylation has not been identified, it seem likely that it will also be the regulatory GTPase of the adenylate cyclase system.

Infection of E. coli with phage T4 leads to a series of structural modifications of the DNA-dependent RNA polymerase of the host bacterium (36). A very fast (after infection) ADP-ribosylation of one of the two α-subunits of the RNA polymerase has been termed alteration (36). The enzyme involved in alteration is relatively non-specific and it ADP-ribosylates a variety of proteins including itself (36). Later during the infectious cycle, another T4 enzyme

(termed the modification enzyme), ADP-ribosylates RNA polymerase at both α-subunits (30, 36). The modification enzyme is more specific than the alteration enzyme. The modified E. coli RNA polymerase cannot transcribe several E. coli genes, which suggests that modification via the ADP-ribosylation of RNA polymerase is involved in the shut-off of host transcription (36, 37). Finally, the virions of another coliphage, N4, contain an ADPR-transferase (88). This enzyme is relatively non-specific; however, it does not modify RNA polymerase (36, 88). The biological role of the N4 phage ADPR-transferase is not known at this time (36, 88).

In general, the ADPR-transferases found in eukaryotic cells are poly-ADPR-transferases (36, 43). For the most part, they appear to be primarily located in the nucleus (36) and may function in the regulation of DNA synthesis (31). However, ADPR-transferases have been found in the mitochondria (55) and in the cytoplasm (76, 93). The biological roles of ADPR-transferases found in these two locations are speculative at this time (36, 43). Moss and Vaughn (76) have recently discovered the presence of an ADPR-transferase in the cytosol of avian erythrocytes. This enzyme differs from all other eukaryotic ADPR-transferase in being a mono-ADPR-transferase. In this way, the enzyme resembles the prokaryotic transferases. Early evidence suggests that this eukaryotic enzyme may function naturally in the cell to regulate adenylate cyclase in a manner similar to cholera toxin (76).

Relatively straightforward methods have been developed to distinguish between mono- and poly-ADPR-transferases (36, 43). The

reaction products of the enzymes are analysed after digestion with snake venom phosphodiesterase. Digestion of poly-ADPR-proteins [$(ADPR)_n$-proteins] yields three different products: one molecule of 5'-AMP from the distal terminus, a number of isoADP-ribose molecules and one molecule of ribose-5-phosphate attached to the acceptor protein (36). In contrast, phosphodiesterase treatment of the reaction products catalyzed by the mono-ADPR-transferases (ADPR-protein) yields only one molecule of 5'-AMP and one molecule of ribose-5-phosphate attached to the acceptor protein (36). No isoADP-ribose is produced.

I. Summary

P. aeruginosa is a complex microorganism with many characteristics consistent with its role as an opportunistic pathogen. The organism is able to persist in environments in which most other pathogens are killed. P. aeruginosa is resistant to many of the antibiotics commonly used to treat other bacterial infections. The organism produces a wide variety of extracellular products that may contribute to its pathogenicity. The product that is most toxic on a weight basis to experimental animals is exotoxin A. Toxin A, like diphtheria toxin, is an ADPR-transferase with EF-2 as the acceptor. In experimental animal models, toxin A produced by P. aeruginosa causes a decrease in the functional EF-2 levels in a variety of organs (most particularly the liver) and contributes significantly to the morbidity and mortality of animals in experiment infection. Its precise role in human infection is not known at this time. In light

of the number of prokaryotic ADPR-transferases, it would not be surprising to find other ADPR-transferases (distinct from toxin A) produced by some strains of P. aeruginosa.

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III. Manuscripts

Paper 1.

Incidence of exotoxin production by Pseudomonas species.

Abstract

Pseudomonas aeruginosa exotoxin A has been shown to catalyze the transfer of the ADP-ribose moiety of NAD^+ onto elongation factor-2, resulting in the inhibition of mammalian protein synthesis. This enzymatic activity (ADPR-transferase) is thought to account for the toxicity of exotoxin A. The distribution of the expression of exotoxin A within Pseudomonas species was examined. Laboratory strains as well as clinical isolates of Pseudomonas aeruginosa were tested. The production of exotoxin A was determined by assaying for ADPR-transferase activity in dialyzed frozen (-20) and thawed cell free supernatants from 22 h cultures or in 10-fold concentrated supernatants. In addition, toxin production was detected immunologically utilizing a modified Elek test. Exotoxin A production was detected in approximately 90% of the 111 isolates of P. aeruginosa. In contrast, none of the other species of Pseudomonas examined produced exotoxin A detectable by either ADPR-transferase activity or immunological reactivity.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen which can cause serious and lethal infections in debilitated or immunosuppressed hosts such as cancer, burn, cystic fibrosis patients and others (17). P. aeruginosa produces a variety of extracellular products which may contribute to its pathogenicity, including hemolysins, proteases, an enterotoxin, and a heat-labile exotoxin originally designated exotoxin A by Liu (15, 18). Exotoxin A has been shown to be more toxic on a

weight basis than the other extracellular products and P. aeruginosa endotoxin (16). Exotoxin A is lethal for mice and dogs (1, 14, 20) and is cytotoxic to tissue culture cells (20).

Exotoxin A has been shown to inhibit protein synthesis when introduced into animals and tissue culture cells (21). Iglewski and Kabat (13) reported that exotoxin A inhibits mammalian protein synthesis by catalyzing the transfer of the adenosine 5'-diphosphate ribosyl (ADPR) moiety of NAD^+ onto elongation factor 2 (EF-2). The resultant ADPR-EF-2 is inactive in protein synthesis (12, 13). This mechanism of action is the same as that found with diphtherial toxin fragment A (5).

The purpose of this study was to develop methods for the in vitro detection of exotoxin A and to employ these methods to determine the distribution of toxin expression in P. aeruginosa and other Pseudomonas species. Two methods were found suitable for this purpose, a modified Elek test (7, 23) and the direct detection of ADPR-transferase activity in cultural supernatants. The results presented in this study indicate that exotoxin A is produced by most strains of P. aeruginosa but rarely if ever by other Pseudomonas species.

Materials and Methods

Pseudomonas strains. Clinical isolates of P. aeruginosa were obtained from patients at Walter Reed Army Medical Center over a 5 year period. Identification of all organisms as P. aeruginosa was by the method of Gilardi (11) as modified by Baltimore et al. (3). The

strains were serotyped using the procedure of Fisher et al. (10) and pyocin typed by the method of Farmer and Herman (8) as modified by Baltimore et al. (3). These strains were tested for toxin production by the fifth subculture. P. aeruginosa designated as laboratory strains included the 18 Farmer ALA pyocin typing strains (kindly provided by B. Minshew, Univ. of Washington), PA-103, PA-464 and W783 (kind gift of P. V. Liu, Univ. of Louisville), PA-01 and PAT-2 (kind gifts of B. Holloway, Monash Univ.), PS-7, 112 and PS-1C (kind gifts of E. Fisher, Portland State Univ.) and NIH 2915 (kind gift of S. Leppla, Frederick, Md.). While many of these strains were originally isolated from patients, they have been maintained in laboratories for up to 20 years and have been subcultured many times. Species of the genus Pseudomonas, other than P. aeruginosa, were isolated, identified, and kindly provided by V. M. Young and M. Moody of the National Cancer Institute (Baltimore, Md.) and by R. A. Finkelstein (Univ. of Texas, Dallas, Texas). Most of these strains were clinical isolates.

Immunological detection of exotoxin A. P. aeruginosa (PA-103) (15) was used to produce exotoxin A which was purified as described previously (18, 24). The purified toxin had a mouse 50% lethal dose (LD_{50}) of 0.25 µg and migrated as a single homogenous protein in SDS-polyacrylamide gels with a molecular weight of 71,500. This toxin was further purified by electrophoresis on conventional polyacrylamide disc gels as previously described (24). The gels were fractionated, the proteins were eluted as previously described (25) and the fraction

eluates were assayed for ADPR-transferase activity as described below. Only those fractions showing peak enzymic activity were pooled and used for immunization of rabbits. Specific antitoxin was obtained by immunization of each rabbit with a total of 50 µg of the purified exotoxin A in complete Freund's adjuvant divided into 3 equal aliquots and injected by the subcutaneous, intramuscular and intraperitoneal routes. The rabbits were boosted in the same manner 2-3 weeks after the primary immunization. The rabbits were exsanguinated 2-3 weeks after the second immunization.

The specificity of the antitoxin was determined by Ouchterlony immunodiffusion analysis against purified exotoxin A and a crude 10X concentrated cultural supernatant from PA-103. The Ouchterlony immunodiffusion assay was a modification of a method by Ouchterlony (19) previously described (9).

The specific antitoxin was used to detect exotoxin A by a modified Elek technique (23). Strips of filter paper (75 x 8 mm) were soaked in the antitoxin and embedded into a petri plate containing trypticase soy broth dialysate, 5% monosodium glutamate, 1% glycerol (15) and 2% noble agar (freshly poured). After the agar had solidified, the test organisms were streaked perpendicular to the filter paper strip as is shown in Fig. 1. Three to five organisms were usually tested per plate, with P. aeruginosa strain PA-103 serving as a positive control. The plates were incubated at 32° C for 48 h at which time they were read for the presence or absence of precipitin lines (Fig. 1). The plates were held at 5° C for 2 to 3 days and

re-read before being discarded. All negative and questionable strains were repeated in duplicate.

ADPR-transferase activity. Pseudomonas strains were grown in a liquid medium consisting of the dialysate from trypticase soy broth, 5% monosodium glutamate and 1% glycerol, described by Liu (15). Ten ml of this inoculated medium in a 500 ml Erlenmeyer flask were incubated at 32° C on a reciprocating shaker (200 linear excursions/min) for 22 h. The cultural supernatant was obtained by centrifugation at 10,000 x g for 45 min. The supernatant was dialyzed against several changes of 0.01 M Tris·HCl buffer, pH 8.0, at 5° C for 24 h. The ADPR-transferase activity of the dialyzed supernatant was potentiated by freezing (-20° C) and thawing the supernatant.

Aminoacyl transferase containing enzymes were prepared from crude extracts of rabbit reticulocytes as described by Allen and Schweet (1) and modified by Collier and Kandell (6). ADPR-transferase activity in cultural supernatants was measured according to the procedure of Collier and Kandell (6) as previously described (13, 25). The assay mixture in a total volume of 65 µl contained 50 mM Tris·HCl pH 8.2, 0.1 mM EDTA, 40 mM dithiotheitol, 25 µl of reticulocyte enzymes, 5 µl (0.367 µM) [adenine-^{14}C] NAD (136 Ci/mol, Amersham/Searle) and 10 µl of cultural supernatant. After 5 min of incubation at 25° C, 65 µl of 10% trichloroacetic acid was added and the precipitates were collected, washed and counted as described previously (13).

All strains that were negative for ADPR-transferase activity by the above method were retested after concentrating the cultural

supernatant ten-fold by ultrafiltration using a PM-10 membrane (Amicon), dialyzing against Tris buffer, and freezing (-20° C) and thawing the concentrated supernatant before assaying for ADPR-transferase activity as described above.

Results

It was necessary to insure the specificity of the antitoxin before using it in our modified Elek technique. Only a single line of precipitation occurred between the pure toxin and antiserum or between the 10X crude supernatant of PA-103 and the antiserum (Fig. 2). Furthermore, a line of complete identity was seen between both the purified and 10X concentrated crude preparations of exotoxin A. Therefore, this antiserum did not detect antigens other than exotoxin A by the Ouchterlony technique and was therefore suitable for detection of this toxin by the Elek test.

The results of the Elek tests are shown in Table 1. Of the 111 *P. aeruginosa* strains examined for exotoxin A production by the Elek tests, 92 gave a positive result. No marked difference was observed between the percentage of positive laboratory strains and the clinical isolates. In contrast, none of the 28 non-aeruginosa *Pseudomonas* strains tested produced exotoxin A as determined by the Elek technique. These strains tested belonged to the following species: *P. fluorescens*, *P. maltophilia*, *P. pseudomallei*, *P. putida*, *P. cepacia*, *P. saccharophila*, and *P. indoloxidans* (Table 1).

Toxin production was also determined by detecting ADPR-transferase activity in supernatants from cultures grown in liquid media (see methods). The process of freezing the supernatants at -20° C and thawing increased the enzymatic activity of the supernatants. A supernatant of PA-103 stored at -70° C typically gave 250 cpm when assayed by ADPR-transferase activity whereas a frozen (-20° C) and thawed aliquot of the same supernatant gave 1,500 cpm. These data, along with additional observations in our laboratory suggest that toxin A is a proenzyme and treatment of a toxin preparation under various conditions activates the ADPR-transferase activity (24). For these reasons, all ADPR-transferase assays were done with frozen (-20° C) and thawed supernatants.

The results of the ADPR-transferase assays are shown in Table 1. Of the 87 P. aeruginosa strains tested, 75 (86%) produced exotoxin A as measured by ADPR-transferase activity. Again, there was no significant difference in the percentage of strains that gave positive ADPR-transferase assays, whether they were clinical isolates or laboratory strains (84% and 92%, respectively). None of the 28 non-aeruginosa Pseudomonas strains tested produced toxin as determined by ADPR-transferase activity.

Out of the first 68 strains that were positive for exotoxin A production by the Elek test, 67 (98%) of these were also positive for toxin as determined by ADPR-transferase activity. Because of this correlation, only Elek negative strains were further tested by the ADPR-transferase assay. A total of 111 isolates of P. aeruginosa were

examined for exotoxin A production by the Elek assay, 19 of which were negative. Eight of these 19 Elek negative strains were toxin positive as determined by ADPR-transferase activity. It is possible that these eight strains produced low amounts of toxin which are not detected by the Elek technique or an alternative explanation is that these strains produce a different serological type of the toxin. These results are not mutually exclusive and are currently being tested in our laboratory. If a toxin positive strain is defined as giving a positive Elek or as positive in the ADPR-transferase assay, then 90% of the P. aeruginosa isolates tested were toxin positive (Table 1).

No correlation was seen between exotoxin A production and the serotype or pyocin type of the strains. However, the 11 strains of P. aeruginosa which were toxin negative (Elek negative and ADPR-transferase activity negative) were of different pyocin types indicating they were indeed 11 different strains of P. aeruginosa and not different isolates of the same strain. All of the nine clinical isolates that were toxin negative were considered primary pathogens in patients that had serious underlying diseases including severe trauma, leukemia with lymphopenia and renal transplants on immunosuppressive therapy. Furthermore, all nine of these patients had unexpectedly benign clinical courses with regards to their P. aeruginosa infections.

Discussion

The role of exotoxin A in the pathogenesis of *P. aeruginosa* infections remains to be clarified. Although evidence is accumulating that this toxin is a major virulence factor of *P. aeruginosa* (2, 4, 13, 22), prior to this report there was a limited amount of information regarding the incidence of toxigenicity among *Pseudomonas* species.

In order to determine the distribution of the expression of toxin in *Pseudomonas* species, it was necessary to develop methods for the *in vitro* detection of exotoxin A. Two different methods to detect exotoxin A by *Pseudomonas* strains were developed. There was a significant positive correlation between these two methods. Of the first 68 strains of *P. aeruginosa* that were positive for exotoxin A production by the Elek test, 67 (98%) of these were also positive for exotoxin A as determined by ADPR-transferase activity. The one isolate that was Elek positive but negative by the ADPR-transferase assay is a possible spontaneous mutant which may produce a cross-reacting non-enzymatically active protein (CRM).

If all strains that give a positive Elek or a positive ADPR-transferase reaction are considered toxinogenic, then 90% of those *P. aeruginosa* strains tested produce exotoxin A. In contrast there were no strains of other *Pseudomonas* species found to produce toxin by these methods.

These data (Table 1) suggest that the ability to produce exotoxin A is widely distributed among *P. aeruginosa* strains and is infrequently

if ever found in other Pseudomonas species. It should be noted that the P. aeruginosa strains were isolated from wounds, urinary tract infections and septicemias. Isolates from burn infections and cystic fibrosis patients have not yet been tested and were not included in this study. It is interesting that there was no correlation observed between the positive or negative strains and any particular source, serotype or pyocin type of P. aeruginosa.

It is possible that the Pseudomonas strains that did not produce exotoxin A as detected by our methods do not represent true negatives. Pseudomonas is known to produce several extracellular proteases; thus, it is possible that these proteases are destroying the toxin before it can be detected by the methods described. Another possible explanation for the failure to detect toxin in all Pseudomonas strains is that the media used for toxin production are not suitable for in vitro toxin production by all strains. In addition, some strains may only produce exotoxin A in vivo or in small quantities that escape detection by the present methods. Alternatively, the negative Pseudomonas strains may be true negatives which are unable to produce exotoxin A.

Studies are in progress in this laboratory to further characterize these "nontoxinogenic" strains and to ultimately determine the role of exotoxin A production in Pseudomonas infections.

While the numbers are small, the unexpectedly benign clinical course seen in the nine patients from whom exotoxin A negative strains of P. aeruginosa were isolated suggests that this toxin may indeed be an important virulence factor in P. aeruginosa infections in man.

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Table 1. Exotoxin A production by *Pseudomonas* species

Species	Number positive/number tested[a]		
	Elek reaction	ADPR-transferase activity	Either assay
P. aeruginosa			
Clinical	69/85 (81%)	51/61 (84%)	76/85 (89%)
Laboratory	23/26 (88%)	24/26 (92%)	24/26 (92%)
Total	92/111 (83%)	75/87 (86%)	100/111 (90%)
Pseudomonas species (non-*aeruginosa*)[b]	0/28 (0%)	0/28 (0%)	0/28 (0%)

[a]Numbers in parenthesis are percentages.

[b]The *Pseudomonas* species tested include: *P. fluorescens* (6 tested), *P. maltophilia* (7), *P. pseudomallei* (6), *P. putida* (6), *P. cepacia* (1), *P. saccharophila* (1), *P. indoloxidans* (1).

Figure 1. Elek plate for the detection of toxinogenicity of Pseudomonas strains. The top and bottom streaks are of tox^+ organisms, while the middle streaks represent a tox^- organism.

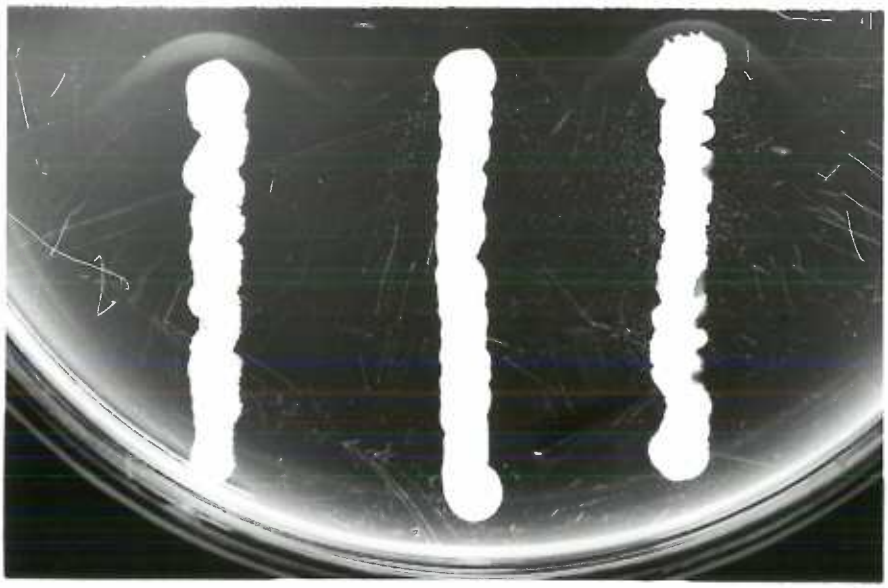
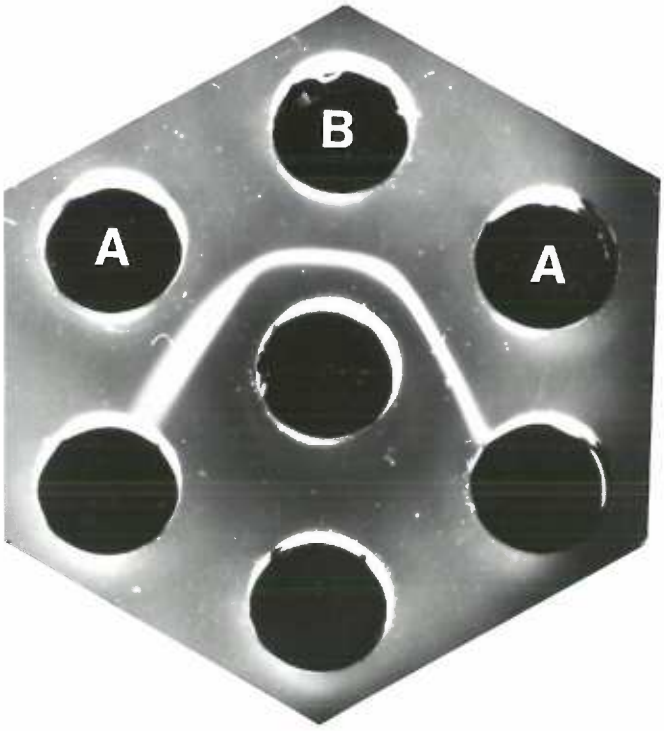


Figure 2. Immunodiffusion analysis of toxin A antiserum. The center well contains the antiserum. Well A contains a 10X concentrated supernatant of strain PA-103. Well B contains purified toxin A.


B
A
A

Paper 2.

The effect of iron on yields of exotoxin A in cultures of *Pseudomonas aeruginosa*.

Abstract

The yields of exotoxin A in Pseudomonas aeruginosa cultures were influenced by the concentration of iron in the culture medium. When the iron concentration of the culture medium was increased from 0.05 μg/ml to 1.5 μg/ml there was at least a 90% decrease in exotoxin A (as measured both by enzymatic activity and by mouse lethality) and a slight increase in the growth of the bacteria. The addition of iron as late as 13 h after initiation of growth represses further measurable increases of exotoxin A within 3 h. Intracellular toxin levels were also reduced by increasing the iron concentrations of the culture medium. The addition of 3.0 μg iron/ml did not significantly alter either the enzyme activity of preformed crude or purified exotoxin A or the mouse toxicity of the pure toxin. Thus it appears that either the rate of production or the rate of intracellular degradation of exotoxin A is regulated by the concentration of iron in the culture medium.

Introduction

Pseudomonas aeruginosa exotoxin A is a potentially important virulence factor (14, 16, 17, 24). Exotoxin A has been purified (4, 12, 18) and its effects characterized (8, 16, 17, 23, 24). Similar if not identical toxins have been found to be produced by approximately 90% of the strains of P. aeruginosa tested (3, 25). Exotoxin A, like diphtheria toxin, inhibits mammalian protein synthesis by catalyzing the transfer of the adenosine 5'-diphosphate-ribose (ADPR) moiety of

NAD^+ (nicotinamide adenine dinucleotide) onto mammalian elongation factor 2 (EF-2) (9-11).

Detectable quantities of exotoxin A were found in some but not in all supernatants from media in which toxinogenic strains of P. aeruginosa were grown (14, 15, 17). Furthermore, individual strains of P. aeruginosa reportedly differ in their requirements for optimal toxin production (17). Since exotoxin A is not formed constitutively by toxinogenic strains of P. aeruginosa it is likely that specific regulatory systems or factors control the synthesis and secretion of exotoxin A in a manner that is distinct from the regulation of bulk protein synthesis.

The present communication describes the effect of iron on the yields of exotoxin A and on the activity of preformed exotoxin A.

Materials and Methods

Reagents. NAD ([^{14}C] adenine) at 280 mCi/mmol was purchased from Amersham/Searle Corp. Dithiotreitol was purchased from Sigma Chemical Co. and lysozyme was purchased from Worthington Biochemical Corporation.

Microorganisms. P. aeruginosa strain PA-103, kindly provided by P. V. Liu (15), was used as a standard toxin producing strain because it produces very little toxin destroying protease (15, 31). Strain PA-103 was stored at -70° C in 12% glycerol.

Exotoxin A. Strain PA-103 was used to produce exotoxin A (15) which was purified as described previously (30). The purified toxin

had a mouse LD_{50} of 0.25 µg/22 g mouse when injected intraperitoneally, and migrated as a single homogenous protein in sodium dodecyl sulfate (SDS) polyacrylamide gels with a molecular weight of 71,500 daltons. Exotoxin A was stored in small aliquots at -70° C.

The specific A antitoxin was prepared against highly purified toxin in rabbits as previously described (3).

Culture medium. The culture medium used was that previously developed by Liu (15). This consisted of the dialysate from trypticase soy broth, .05 M monosodium glutamate and 1% glycerol. The medium was deferrated to obtain an iron concentration of 0.05 µg/ml by adding 2 ml/liter of 50% $CaCl_2 \cdot 2H_2O$, boiling for 5 min in a water bath and filtering through Whatman number 1 filter paper to remove precipitate (21). Residual iron in the medium was determined as described by Mueller and Miller (21). To obtain know concentrations of iron in the deferrated medium, standard sterile solutions of $FeSO_2 \cdot 7H_2O$ were added.

Culture flasks were acid cleaned and rinsed with 20 changes of deionized water. Unless otherwise stated, 50 ml of medium was added to a 1 liter Erlenmeyer flask (flask to volume ratio of 20:1) and inoculated with 0.1 ml of a 15 h culture of PA-103. The flasks were incubated at 32° C on a reciprocating shaker (150 linear excursions/min) (Lab-line Instruments, Melrose, Ill.). Optical densities were obtained by aseptically removing an aliquot of the culture and reading the OD_{540} in a Beckman spectrophotometer 20.

The culture supernatants were obtained by centrifugation at 10,000 x g for 20 min. The supernatants were filter sterilized, immediately frozen and stored at -70° C. Both the animal toxicity and the enzymatic activity of toxin A was stable for at least 4 mo. when the toxin was stored at -70° C.

Adenosine 5'-diphosphate-ribose transferase (ADPR-transferase) activity. Aminoacyl transferase containing enzymes were prepared from crude extracts of rabbit reticulocytes as described by Allen and Schweet (1) and modified by Collier and Kandel (6). The ADPR-transferase activity of cultural supernatants and purified exotoxin A were measured according to the procedure of Collier and Kandel (6) modified exactly as described previously (3). Exotoxin A is found in culture supernatants as a proenzyme and treatment with a denaturing agent and a reducing agent activates the ADPR-transferase activity of exotoxin A (12, 30). In the current study the ADPR-transferase activity was measured on culture supernatants both before and immediately after treatment with 4 M urea and 1% dithiotreitol (DTT) as previously described (30).

Mouse lethality. Female Swiss-Webster mice (14-17 g) were used for determining the mean lethal dose (LD_{50}) of toxin preparations (27). These toxin preparations were diluted in sterile physiological saline, pH 7.8 and one ml aliquots were injected intraperitoneally into each of at least 4 mice.

Solid Phase Radioimmune Inhibition Assay. Exotoxin A was quantified by a modification of a solid phase radioimmune inhibition assay

originally described by Zollinger and Mandrell (33). The wells of polyvinyl flexible "U" microtiter plates (Cooke Laboratory Products Inc., Alexandria, Va.) were coated with purified exotoxin A diluted in Dulbecco's phosphate buffered saline (PBS) (Grand Island Biological Co., Grand Island, N.Y.) to a concentration of 50 µg/ml by placing 25 µl of diluted antigen in the wells and allowing the antigen to bind for 1 h at 37° C. Following aspiration of unbound antigen the wells were rinsed with 50 µl of filler (10% fetal calf serum, 0.2% sodium azide and 0.02% phenol red in PBS pH 7.4) followed by addition of 100 µl of filler and incubation at 37° C for 1 h. The solution was then dumped out and the wells washed twice with PBS. Inhibition mixtures consisting of 50 µl of exotoxin A antisera diluted 1:8000 in filler and 50 µl of various dilutions in filler of unknown or standard exotoxin A samples were placed in the well of a separate microtiter plate and incubated at 37° C for 1 h. The final concentration of exotoxin A antisera corresponded to approximately 50 ng antitoxin/well as previously determined by a direct solid phase binding assay (32). Controls consisted of filler only and filler plus antitoxin. After incubation, duplicate 20 µl samples of the mixtures were placed in the antigen coated wells of the flexible microtiter plate. Binding was allowed to proceed overnight at room temperature, the liquid was aspirated and the wells washed once with filler and five times with PBS. Goat anti-rabbit immunoglobulin (Antibodies Inc., Davis, Ca.) was further purified and labeled with ^{125}I (^{125}I-GARG) as described by Zollinger et al. (32). ^{125}I-GARG (25 µl) was placed in each well and

allowed to bind overnight at room temperature. The liquid was aspirated and the wells washed once with filler and four times with PBS. The wells were then cut off into tubes and counted as previously described (32). The concentration of exotoxin A in each sample was determined from a standard curve obtained using known amounts of pure exotoxin A. The percent inhibition was calculated by the formula:

$$\text{Percent Inhibition} = 100 - \frac{\text{mean cpm bound with inhibitor-background} \times 100}{\text{mean cpm bound without inhibitor-background}}$$

Antigen coated wells that received filler only were used as the background controls. The uninhibited controls received an inhibition mixture which contained filler instead of toxin samples. As shown in Fig. 1 the standard curve was linear when from 0.04 to 0.2 μg exotoxin A/ml is used in the competition mixtures.

Extraction of intracellular toxin. Two methods, sonication and lysis of spheroplasts, were used to extract intracellular toxin from strain PA-103. In both procedures the cells from 22 h cultures were extensively washed with 0.01 M Tris buffer, pH 8.0 (Sigma). Washed cells were resuspended in 10 ml of Tris buffer, and disrupted by sonication with ten 30 sec bursts (50% maximal output) using a Biosonik IV (Bronwill) sonicator. Each burst was followed by a 2 min incubation in an ice bath. The above treatment resulted in the destruction of greater than 90% of the cells as determined by light microscopy. The cellular debris was removed by centrifugation at 10,000 x g for 45 min and the resulting intracellular extract was filter sterilized and stored at -70° C.

Ethylenediaminetetraacetate-lysozyme spheroplasts of PA-103 were obtained using the procedure of Birdsell and Cota-Robles (2) with the following modifications: lysozyme was added to a concentration of 40 µg/ml and the cells were incubated at room temperature for 15 min. The osmotically fragile spheroplasts were lysed by the addition of cold distilled-deionized water. The resulting intracellular extract was clarified by centrifuging at 10,000 x g for 45 min to remove cellular debris, filter sterilized and stored at -70° C.

Protein determinations. Protein was determined by a modification of the Lowry method (13). Bovine serum albumin was used as the standard.

Results

Effect of iron on yields of extoxin A. Strain PA-103 was grown for 22 h in medium containing various iron (ferrous sulfate) concentrations. The yield of exotoxin A as measured by its enzymatic activity was maximal in cultures containing 0.05 µg iron/ml. Concentrations of iron below 0.05 µg/ml were not generally obtained using the $CaCl_2$ deferration procedure. Toxin A yields might have increased further in medium containing less than 0.05 µg iron/ml. Increasing the concentration of iron from 0.05 µg/ml to 0.1 µg/ml decreased the exotoxin A yield (Fig. 2). The toxin yield in a culture containing 1.5 µg/ml was less than 10% of that ina culture containing 0.05 µg iron/ml. The inhibitory effect of iron on exotoxin A yields began to level off at concentrations of iron greater than 1.0 µg/ml and small amounts of toxin activity could still be detected in supernatants of cultures which contained 10 µg iron/ml.

Increasing the iron concentration of the growth medium also decreased the mouse lethality of the supernatants of such cultures. The mouse LD_{50} of a culture supernatant containing 0.05 µg iron/ml was contained in 1 ml of a 1:75 dilution, whereas the LD_{50} of a culture containing 3.0 µg iron/ml was contained in 1 ml of a 1:15 dilution (Table 1). That the toxicity of the culture supernatants was due to exotoxin A was shown by the ability of specific antitoxin to neutralize this toxicity (Table 1). The small amount of residual toxicity of the antitoxin treated crude supernatant was probably due to other extracellular products known to be produced by P. aeruginosa (16).

Effect of iron on the rate of growth and exotoxin A release. Strain PA-103 was grown in medium under two different iron (ferrous sulfate) concentrations, .05 µg/ml and 1.5 µg/ml (Fig. 3). Increasing the iron concentration of the medium had no significant effect on the growth rate of PA-103 through the first 19 h of culture. The culture grown in medium containing 0.05 µg iron/ml entered maximum stationary phase at 19 h whereas the culture grown in medium containing 1.5 µg iron/ml continued to increase through 24 h resulting in a slightly larger final yield of bacteria.

The earliest time at which exotoxin A could be detected in the culture grown in medium containing 0.05 µg iron/ml was 9 h (Fig. 3). The concentration of exotoxin A continued to increase in this culture through 19 h. Exotoxin A was not detectable until 11 h in the culture grown in medium containing 1.5 µg iron/ml and both the rate of release as well as the final yield of exotoxin A was markedly decreased as compared to the culture grown in medium containing 0.05 µg/ml (Fig. 3).

Rate of toxin release following the addition of iron to low iron growth media. Strain PA-103 was grown in low iron (0.05 µg/ml) growth medium. After 13 h of growth, the culture was split into two separate flasks. To one flask enough ferrous sulfate was added to result in a final concentration of 1.5 µg iron/ml. No additional iron was added to the second flask. The cultures were returned to the shaker and aliquots were removed at hourly intervals and assayed for exotoxin A enzyme activity. There was a lag period of 3 h before a significant difference in the enzyme activity of the two cultures was observed (Fig. 4). At this point the concentration of exotoxin A leveled off in the 1.5 µg iron/ml culture but continued to increase in the 0.05 µgm iron/ml culture. It should be noted that the enzyme activity of the culture grown in medium containing 1.5 µg iron/ml while not increasing after 15 h does remain constant through 24 h and does not decrease as might occur if protease activity was increasing in the presence of added iron.

Effect of iron on the biological activity of preformed exotoxin A. While the stability of the enzyme activity of exotoxin A released into the medium containing 1.5 µgm iron/ml (Figs. 3 and 4) suggested that iron was not affecting the biological activity of preformed exotoxin A, this possibility was tested further by adding iron to preformed exotoxin A. The addition of 3 µg/ml iron had no significant effect on the enzymatic activity of either crude or purified toxin (Table 2). The enzymatic activity of exotoxin A has been shown to be potentiated by treatment with a combination of denaturing and reducing agents (12,

30). Iron had no effect on the ability of urea and DTT to potentiate the enzymatic activity of exotoxin A, nor did the addition of iron effect the mouse lethality of preformed exotoxin A (Table 2).

Effect of increasing iron concentrations in the culture medium on the intracellular toxin concentration. It is possible that the effects of iron are due to changes in the transport of toxin out of the cell. In this case, it might be expected that the intracellular concentration of toxin would either remain essentially constant or increase as the iron concentration in the growth medium was increased. To test this, we sonicated washed cells of PA-103 that were grown in medium supplemented with various concentrations of iron and measured the enzyme activity of the cell lysate. Increasing the concentration of iron in the growth medium resulted in a decrease in the intracellular exotoxin A activity (Table 3).

An unexpected result of this experiment was that the intracellular toxin appeared to be in an altered enzymatic form. Thus the activity of the intracellular toxin was not increased by treatment with urea and DTT (Table 3). As previously reported (30), the enzyme activity of culture supernatants of PA-103 was increased by treatment with a denaturant (4 M urea) and a reducing agent (1% DTT). In contrast, the enzyme activity of the intracellular toxin obtained by sonication was actually decreased by treatment with these agents. Furthermore, when the intracellular toxin was obtained by a completely different method (lysis of spheroplasts) it again was in the active form (data not shown). The enzymatic activity of the intracellular toxin, like that

of extracellular toxin, was completely neutralized by preincubation with specific anti A toxin antibody (data not shown). In order to quantitate intracellular toxin A independent of its enzymatic activity a solid phase radioimmune inhibition assay as described in methods was utilized. Increasing the iron concentration of the culture medium from 0.05 to 0.5 µg/ml decreased the intracellular toxin from 0.16 µg/ml to 0.8 µg/ml, and when the iron concentration was 5.0 µg/ml, exotoxin A protein could not be detected in the cell sonicates (Table 3).

Discussion

Liu (15) reported that a number of factors, such as aeration, 32° C and the presence of glycerol, were required for maximal yields of exotoxin A. He also reported that nucleic acids inhibited production of exotoxin A but enhanced the growth of P. aeruginosa strain PA-103 (15). Furthermore, individual strains of P. aeruginosa differed in their requirements for optimal toxin production (17). Thus exotoxin A is not formed constitutively by toxinogenic strains of P. aeruginosa.

This report describes another factor which influences the yield of exotoxin A in cultures of P. aeruginosa PA-103. We have shown that the yield of exotoxin A is influenced by the concentration of iron in the culture medium. The toxin yield was greatest under conditions of low iron (approximately 0.05 µg/ml) in the culture medium (Fig. 2). Strain PA-103 grown in medium supplemented with increasing concentrations of iron yielded proportionally less toxin (Figs. 2, 3 and Table 1). The rate of release of exotoxin A into the culture medium was also greater

in cultures grown in low iron medium as compared to those grown in medium containing added iron (Fig. 3). Furthermore, adding iron to a culture of PA-103 already producing toxin inhibited further increases in extracellular toxin levels within 3 h (Fig. 4).

Intracellular levels of exotoxin A as measured in a solid phase radioimmune competition assay were also reduced by growing strain PA-103 in culture medium containing increasing concentrations of iron (Table 3). This assay was particularly useful since it detected small amounts of toxin (0.04 μg/ml) and the standard curve obtained with pure toxin was the same regardless of the enzymatic form of the toxin. Thus the standard curve was unaltered when pure exotoxin A was or was not treated with urea and DTT to convert it to its enzymatically active form (J. Sadoff, Walter Reed Army Institute of Research; unpublished observations). This proved important in the current study since the biological activity of the intracellular toxin was different from that of the extracellular toxin. Thus the intracellular toxin was enzymatically active (Table 3), whereas the extracellular toxin (as previously reported [12, 30]) required treatment with urea and DTT to convert it from its proenzyme form to its enzymatically active form. Since the intracellular extract was obtained using sonication, it was possible that the sonication in some way "activated" the intracellular toxin. While this possibility cannot be totally ruled out, two lines of evidence tend to discount it. First, the enzyme activity of supernatants that were sonicated in the same manner as the cells could still be increased by treatment with urea and DTT and second, the intracellular

toxin obtained by a completely different method (lysis of spheroplasts) was in the active form (data not shown). The possibility that the bulk of intracellular toxin A is in the enzymatically active, non-lethal form (12, 3) suggests that the structure of toxin A may somehow be altered during or after secretion from the bacterial cell.

The addition of iron to preformed exotoxin A did not alter its mouse toxicity, reduce its enzymatic activity or convert the toxin from its proenzyme form to its active enzyme form (Table 2). Thus it appears likely that increasing the iron content of the culture medium reduced the yield of exotoxin A by either decreasing the rate of production or increasing the rate of intracellular degradation of toxin.

The final yield of bacteria was slightly increased in high iron cultures compared to low iron cultures (Figs. 2 and 3). However, no significant effect of iron could be seen on the rate of bacterial growth through 19 h of culture (Fig. 3). Furthermore, although the iron effect on exotoxin A yields was quite reproducible the effect on bacterial growth varied somewhat from day to day. Although the studies reported here were carried out using the ferrous iron (in the form of $FeSO_4 \cdot 7H_2O$), we have obtained similar results using the ferric ion.

Diphtheria toxin and exotoxin A from P. aeruginosa have a similar if not identical mode of action. Both toxins catalyze the transfer of the adenosine 5'-diphosphate-ribosyl moiety of NAD^+ onto EF-2 (5, 9-11). This covalent modification of EF-2 renders it unable to cause translocation of ribosomes along messenger RNA (mRNA) during protein synthesis (5). It has long been known that the production of diphtheria toxin

(7, 19, 20, 22, 26, 28) is regulated by the concentration of iron in the growth media. It has also been reported that the production of Shigella dysenteriae toxin is regulated by the concentration of iron in the culture medium (29). This study extends the effect of iron on toxin yields to P. aeruginosa exotoxin A.

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Table 1. LD_{50} values of supernatants from cultures grown in medium containing 0.05 and 1.5 µg of iron per ml

Conc of iron in medium (µg/ml)	Treatment[a]	LD_{50} (dilution of supernatant)
0.05	None (saline)	1:75
	NRS[b]	>1:100
	Antitoxin	1:4
1.5	None	1:15

[a]One part of supernatant was mixed with one part of saline, normal rabbit serum, or antitoxin and incubated for 5 min at 37° C before being injected into mice.

[b]Normal rabbit serum. We have consistently found that the addition of normal serum or bovine serum albumin enhances the toxicity of toxin A (probably by stabilizing the toxin molecule).

Table 2. Effect of iron on the activity of preformed toxin

	Enzyme activity[b]	Mouse LD_{50} (µg)
Crude supernatant prepn[a]		
Untreated	1.0	
Supernatant + Fe	1.05	
Supernatant + urea, DTT	8.6	
Supernatant + Fe, urea, DTT	8.3	
Pure toxin prepn[a]		
Untreated	1.0	0.25
Toxin + Fe	0.9	0.25
Toxin + urea, DTT	4.0	
Toxin + Fe, urea, DTT	3.7	

[a]Iron was added to give a final concentration of 3.0 µg/ml.

[b]Enzyme activities are based on the acid-insoluble radioactivity counts per min from the ADPR-transferase assay, where counts per min from the assay of the untreated toxin or supernatant preparation are equivalent to 1.0 activity units.

Table 3. Effect of increasing concentrations of iron in the culture medium on the intracellular toxin A concentration[a]

Conc of iron in medium (µg/ml)	ADPR-transferase activity (CPM)		Exotoxin A (µg/ml)[b]
	Untreated[c]	Treated[d]	
0.05	236	41	0.16
0.5	89	0	0.08
2.5	87	0	
5.0			ND[e]
10.0	0	0	

[a]The lysates were adjusted to a protein concentration of 260 µg/ml.

[b]Obtained by using the solid-phase radioimmune inhibition assay described in the text.

[c]Counts per minute of untreated portion.

[d]Counts per minute of portion treated with urea and DTT.

[e]ND, Not detectable.

Figure 1. Solid phase radioimmune competition assay: standard curve obtained with exotoxin A. (●) Percent inhibition of binding of ^{125}I-GARG in the presence of increasing concentrations of pure exotoxin A.

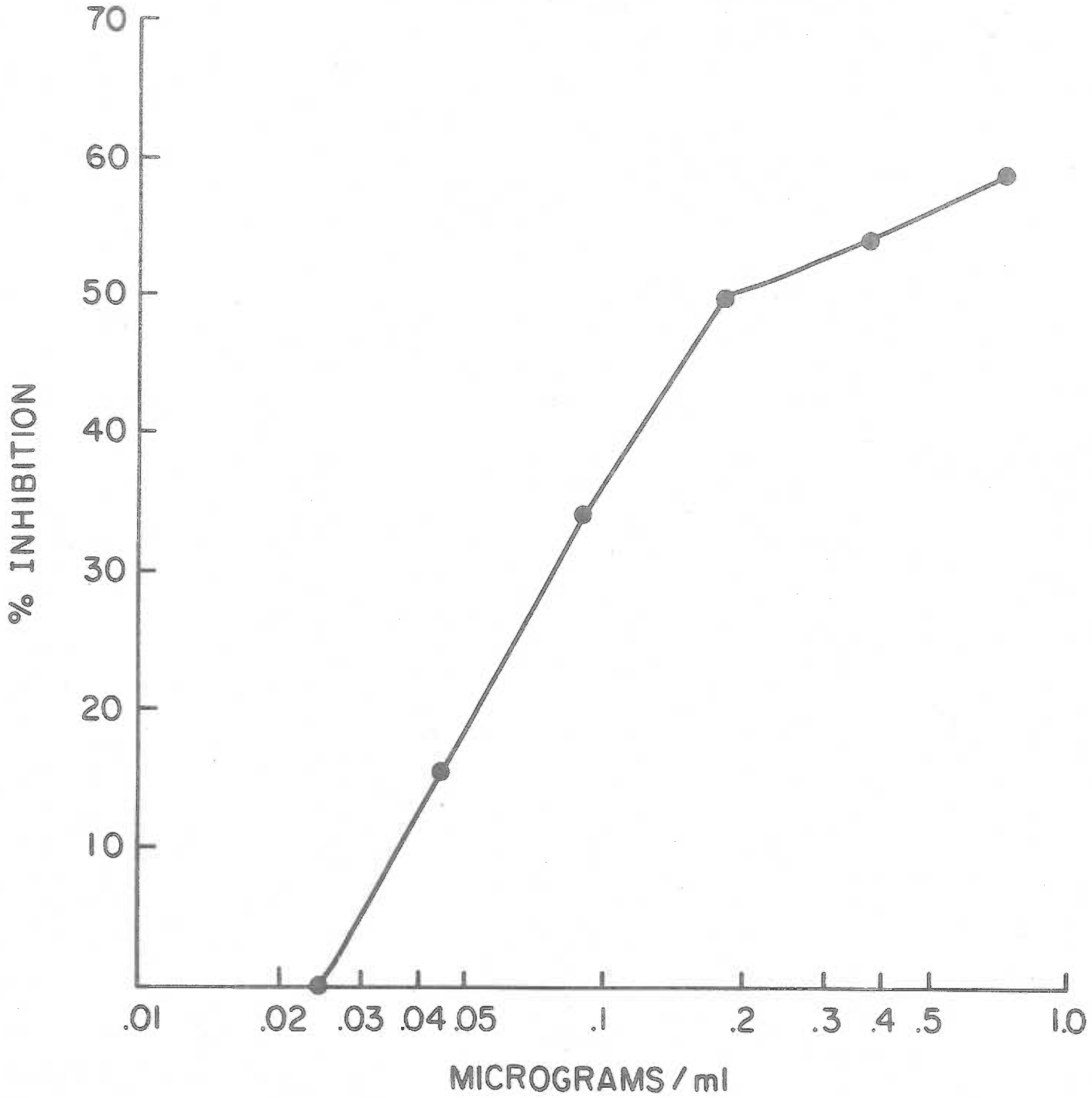

70
60
50
40
30
20
10
% INHIBITION
.01
.02
.03
.04
.05
.1
.2
.3
.4
.5
1.0
MICROGRAMS / ml

Figure 2. Effect of increasing concentrations of iron on the yield of exotoxin A in cultures of P. aeruginosa PA-103. CPM x 10^{-2} is the acid insoluble radioactivity counts per min from the ADPR-transferase assay on activated (urea + DTT treated) samples. Symbols: (●) ADPR-transferase activity (CPM x 10^{-2}). (○) Bacterial growth as measured by OD_{540}.

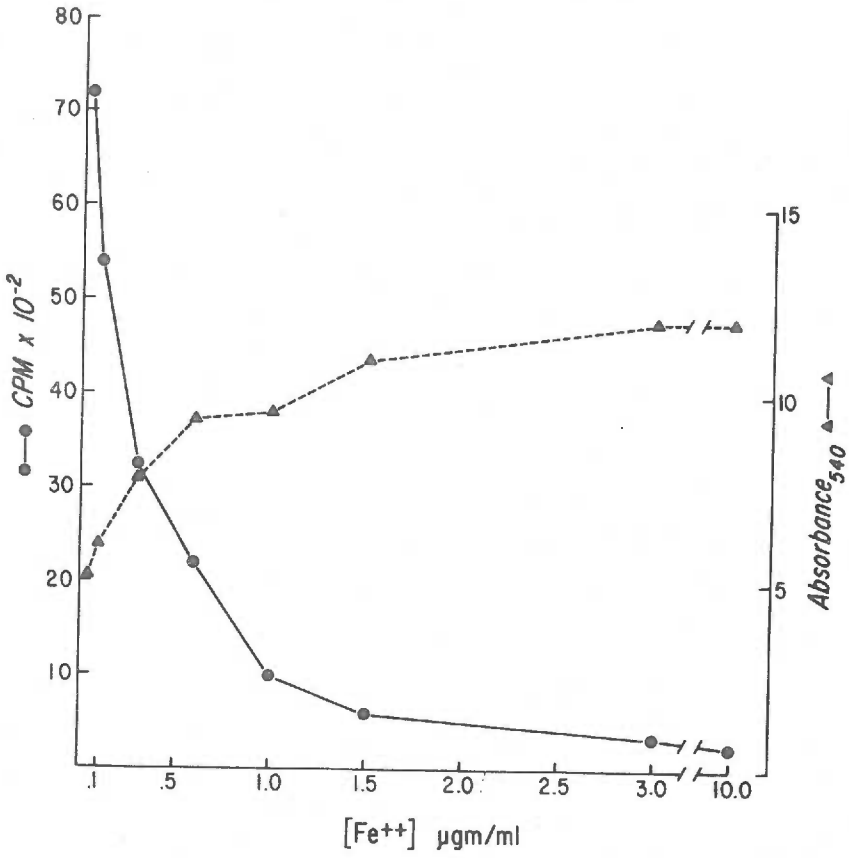

80
70
60
50
40
30
20
10
CPM x 10⁻²
15
10
5
Absorbance 540
.1
.5
1.0
1.5
2.0
2.5
3.0
10.0
[Fe++] µgm/ml

Figure 3. Effect of iron on the rate of growth and exotoxin A release. Symbols: (●) ADPR-transferase activity (CPM x 10^{-2}) of PA-103 supernatants in low (0.05 µg/ml) iron medium. (○) ADPR-transferase activity in high (1.5 µg/ml) iron medium. Bacterial growth as measured by OD_{540} in low (▲) and high (△) iron medium.

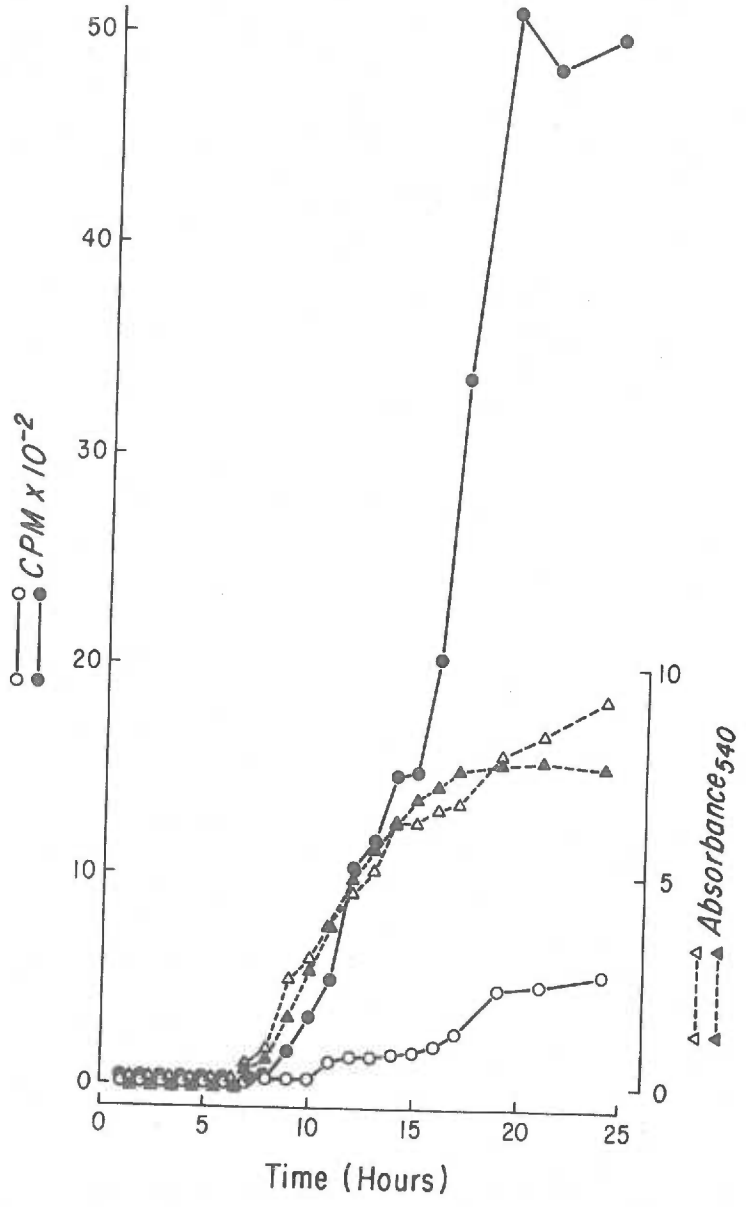
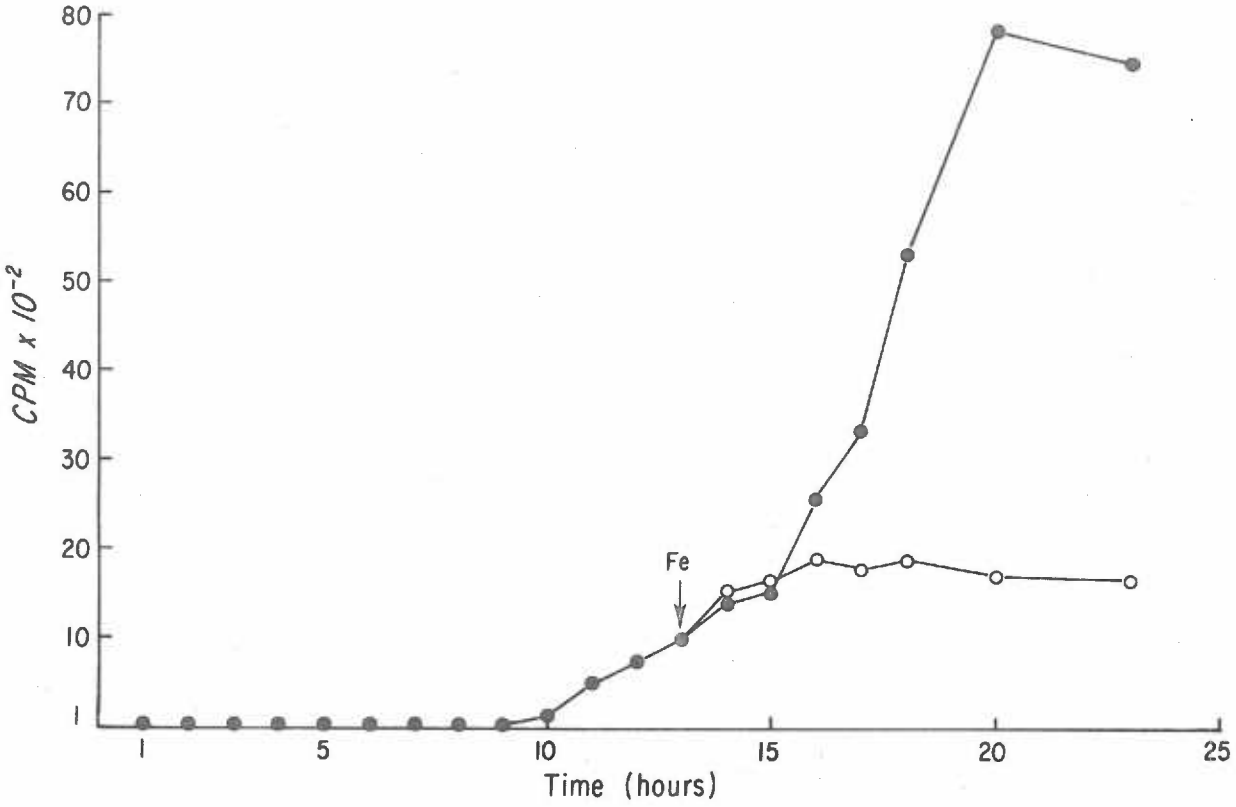

50
40
30
20
10
0
CPM x 10⁻²
10
5
0
Absorbance 540
0
5
10
15
20
25
Time (Hours)

Figure 4. The rate of exotoxin A release after the addition of iron to low (0.05 µg/ml) iron medium. The culture was split into two separate cultures at 13 h and iron was added to one flask to give a concentration of 1.5 µg/ml. Symbols: ADPR-transferase activity (CPM x 10^{-2}) in cultures grown in (●) low iron (0.05 µg/ml) and (○) high iron (1.5 µg/ml) medium.


80
70
60
50
40
30
20
10
1
CPM x 10⁻²
Fe
1
5
10
15
20
25
Time (hours)

Paper 3.

Influence of iron on yields of extracellular products in cultures of *Pseudomonas aeruginosa*.

Abstract

The effect of the iron content of the medium on the yields of extracellular products by seven distinct strains of P. aeruginosa was examined. All strains showed at least an 85% decrease in toxin A yields when grown in medium containing 5.0 µg iron/ml (high iron) as compared to 0.05 µg/ml (low iron), while bacterial growth increased approximately two-fold. During the course of examining extracellular products produced by P. aeruginosa we found many strains that produced an extracellular factor which agglutinated red blood cells. This hemagglutinin (HA) was non-dialysable, heat stable and resistant to pronase and trypsin. The effect of iron on extracellular yields of HA was strain dependent; four of seven strains showed decreases in HA yields in high iron medium. Similarly, the effect of increasing the iron concentration of the growth medium on yields of total extracellular proteases or on elastase was strain dependent. The amount of total extracellular protein was decreased by at least 31% in the high iron medium for all strains of P. aeruginosa examined. Detailed studies on one strain (WR-9) showed that in the presence of increasing amounts of iron in the medium, the extracellular yields of toxin A, protease and HA were decreased in a similar manner. In addition, the rates of release of these extracellular products were similar at a given iron concentration. Thus it appears that the yields of other extracellular products of P. aeruginosa besides toxin A are influenced by the concentration of iron in the growth medium.

Introduction

The presence of excess iron in the culture medium has been shown to inhibit yields of diphtheria toxin (19, 23, 26), Shigella dysenteriae type 1 toxin (5, 29) and Pseudomonas aeruginosa toxin A (1). These toxins are produced at maximal levels late in the bacterial growth cycle when iron is growth limiting (1, 6, 27).

Although the structural gene for diphtheria toxin is located on the DNA of toxin$^+$ corynebacteriophage the physiological state of the host bacterium has been shown to influence toxin production (13, 22, 24). Thus Kanei et al. (13) isolated bacterial mutants that produced diphtheria toxin at the normal rate in medium containing excess iron. It is generally believed that in C. diphtheriae the iron effect is specifically restricted to the toxin gene product because phage production continues in the presence of excess iron (11).

The location of the structural gene for Pseudomonas toxin A is presently unknown. However, the deleterious effect of iron on yields of extracellular products of P. aeruginosa is not restricted to toxin A. Yields of the non-proteinaceous pigments fluorescein and pyocyanine reportedly decreased with increasing concentrations of iron in the growth medium (3, 10, 14, 28).

Although the relationship between iron and the yields of bacterial toxins has been the subject of numerous studies (1, 5, 19, 23, 26, 29) the molecular mechanism(s) underlying iron regulation remains unknown. The current study was undertaken to extend our previous results on toxin A yields by strain PA-103 (1) to different strains of P.

aeruginosa and to determine if the iron concentration of the growth medium influences the extracellular yields of other P. aeruginosa products such as proteases and hemagglutinin.

Materials and Methods

Microorganisms. P. aeruginosa strain PA-103, originally isolated by Liu (16), has been extensively characterized (25). The other P. aeruginosa strains utilized in this study (WR 4, 9, 27, 28, 35, 56) were kind gifts of J. Sadoff, Walter Reed Army Institute of Research, Washington, D.C. The organisms were serotyped (8) using antisera prepared and kindly provided by Dr. M. Fisher, Parke Davis Co., Detroit, Mich. Pyocin typing was performed using the indicator strains and method of Farmer and Herman (7).

Reagents. NAD ([^{14}C] adenine) at 280 mCi/mmole was purchased from Amersham-Searle Corp. Dithiotreitol, elastin-congo red, crystalline bovine serum albumin and casein (technical grade) were purchased from Sigma Chemical Co. and bovine gamma globulin from Bio-rad Laboratories.

Medium and culture conditions. The culture medium consisted of trypticase soy broth dialysate, 1% glycerol, and 0.05 M monosodium glutamate previously described by Liu (17). The medium was deferrated (21) and the residual iron concentration was determined by the procedure of Mueller and Miller (21) as previously described (1). To obtain known concentrations of iron in the deferrated medium, standard sterile solutions of $FeSO_4 \cdot 7H_2O$ were added.

Culture flasks were acid cleaned and rinsed with 20 changes of deionized water. Unless otherwise stated, 10 ml of medium was added to a 125 ml Erlenmeyer flask and inoculated with 0.1 ml of a 15 h shaking culture of the appropriate P. aeruginosa strain. The flasks were incubated at 32° C in a reciprocating shaker (150 linear excursions/min) (Lab-line Instruments, Melrose, Ill.) for 22 h. Bacterial growth was measured by aseptically removing a portion of the culture and reading the optical density at 540 nm in a Beckman Spectrophotometer 20. The cultural supernatants were obtained by centrifugation at 10,000 x g for 20 min. The supernatants were dialysed against 0.01 M Tris (hydroxymethyl)aminomethane-hydrochloride (Tris) buffer, pH 8.0, at 5° C for approximately 18 h, then stored at -70° C.

ADPR-transferase activity. Aminoacyl transferase containing enzymes were prepared from extracts of wheat germ as described by Chung and Collier (4). The ADPR-transferase activity of activated (urea + DTT treated) supernatants was measured as previously described (30). To quantify toxin A based on its enzymatic activity, standard curves were obtained daily with pure toxin and the amount of toxin A present in a crude supernatant calculated from the standard curves (12). Triplicate samples in the ADPR-transferase assay did not vary by more than 10%.

Protease assays. Total proteolytic activity in the crude supernatants of cultures of P. aeruginosa was determined by the method of Kunitz (15) as modified by Wretlind and Wadstrom (31) using casein as the substrate. In order to minimize variation a single batch of casein was purchased and used throughout this study. Triplicate samples in the protease assay did not vary by more than 15%.

Elastase activity was quantified using elastin-congo red as a substrate. The reaction mixture consisted of Tris-maleate buffer (0.1 M, pH 7.0) supplemented with $CaCl_2$ (1 mM) (buffer A). One ml of culture supernatant was added to 2 ml of buffer A containing 10 mg elastin-congo red. The reaction was carried out in stoppered 15 ml centrifuge tubes incubated 2 h in a 37° C water bath with rapid shaking. The reaction was terminated by the addition of 2 ml sodium phosphate buffer (0.7 M, pH 6.0). The precipitate was removed by centrifugation. The blank consisted of 3 ml buffer A containing 10 mg elastin-congo red. Elastase activity was determined by reading absorbance of the supernatants at 495 nm in a Beckman Spectrophotometer 20. Triplicate samples in the elastase assay did not vary by more than 15%.

Hemagglutinin (HA) assay. Fresh sheep erythrocytes stored in Alsevers solution for 3-5 days were washed 3 times with at least 20 cell volumes of the following buffer (buffer B): 0.082 M NaCl, 0.043 M $Na_2HPO_4 \cdot 2H_2O$, 0.0107 M KH_2PO_4, pH 7.4. The washed cells were formalinized using the method of Galazka and Abgarowicz (9). Equal volumes of 10% washed sheep cells (in buffer B) and 3% formalin were mixed and stirred at 37° C for 18 h. The cells were centrifuged, washed 5 times in 2.5 volumes of buffer B, resuspended to a 10% concentration in buffer B containing merthiolate (1:10,000) and stored for up to 2 mo. at 4° C. Just prior to use the cells were washed and resuspended in buffer B containing merthiolate (1;10,000) to a final concentration of 0.2%. HA assays were performed in plastic microtiter plates with V-bottom wells (Linbro Chemical Co., Inc., New Haven, Conn.). Serial two-fold dilutions (in buffer B containing 1% bovine

serum albumin and 1:10,000 merthiolate) of P. aeruginosa supernatants were made and 50 µl of each dilution added to the wells of a microtiter plate. Next, 50 µl of a 0.2% suspension of formalinized sheep cells were added to each well. The plates were incubated at 25° C for 24 h and examined for HA activity. The reciprocal of the highest dilution that showed complete hemagglutination was considered the HA titer. Formalinized SRBC were utilized to assay for the HA because they were much more stable than non-formalinized SRBC and they were resistant to the action of P. aeruginosa hemolysins (19) which may have otherwise made interpretations of the results difficult.

Protein determination. Protein was determined by the method of Bradford (2) modified by using a commercial reagent, Bio-Rad Protein Assay Dye Reagent Concentrate, purchased from Bio-Rad Laboratories, Richmond, Ca. Bovine gamma globulin was used as the standard.

Results

Characterization of P. aeruginosa strains. The P. aeruginosa strains used in this study were originally isolated from human infections and differed from one another in serotype or pyocin type (Table 1). These isolates were chosen for this study because they represented seven distinct strains of P. aeruginosa which produced detectable quantities of toxin A in vitro. The decision to utilize these strains was made independently of their ability to produce proteases, elastase or hemagglutinin (HA) activity. However, all seven of the strains

produced proteases and HA and six of seven strains produced elastase (Table 1).

Growth in high and low iron medium. The P. aeruginosa strains were grown in deferrated medium containing 0.05 µg iron/ml (low iron) and in deferrated medium to which iron was added to give a final concentration of 5.0 µg/ml (high iron). All seven of the strains grew to a higher cell density (approximately 2-fold) in the high iron medium as compared to the low iron medium (Table 2).

Toxin A yields in high and low iron medium. The yields of toxin A in supernatants from cultures of the seven strains of P. aeruginosa grown in high and low iron medium were examined. The quantity of toxin A in the supernatant fluids was calculated from standard curves obtained by assaying known ng amounts of pure toxin A (Fig. 1). Using our previously reported assay system which included a 5 min incubation period (30) we were able to detect 4 ng of toxin A (400 ng/ml) (Fig. 1A). The sensitivity of the assay system was increased to 0.5 ng toxin A (50 ng/ml) by extending the incubation period from 5 to 30 min (Fig. 1B). Because the quantity of toxin A varied from strain to strain and with the concentration of iron in the medium it was necessary to assay aliquots of each sample under both incubation conditions.

The yields of toxin A in the high iron medium were decreased as compared to yields in the low iron medium for all 7 of the P. aeruginosa strains examined (Table 2). Strain PA-103 produced the highest yield of toxin A, which is not surprising since this strain was originally selected by Liu for this property (16). The other 6 strains

tested produced less toxin A than PA-103 even when grown in the low iron medium (Table 2). Thus strain WR-9 produced 1/5 and WR-56 1/60 as much toxin A as did strain PA-103 (Table 2). However, like PA-103 the yields of toxin A by these 6 strains were decreased when the iron concentration of the growth medium was increased (Table 2). The extent of inhibition of A yields in the high iron medium appeared to be at least 85% for all seven of the P. aeruginosa strains tested. Results similar to these (Table 2) have been obtained using a reversed passive hemagglutination assay for toxin A which measured toxin A independent of its enzymatic activity (data not shown).

Effect of iron on protease yields. To determine if the concentration of iron in the growth medium affects the yields of other extracellular products of P. aeruginosa, we examined the effect of iron on total protease yields. The amount of extracellular protease produced in low iron medium varied from strain to strain and was as high as 13.3 proteolytic units/ml for strain WR-4 and as low as 0.3 proteolytic units/ml for strain PA-103 (Table 3A).

In contrast to the uniform decrease in toxin A yields (Table 2) the effect of increasing the iron content of the medium on protease yields varied from strain to strain (Table 3A). Six of the seven strains examined showed a detectable decrease in protease yields when grown in high iron medium as compared to low iron medium (Table 3A). These decreases varied over a range of 24% to 96%. The other strain, WR-27, showed an increase in the yield of extracellular protease when grown in medium containing the higher concentration of iron (Table 3A).

Thus, P. aeruginosa strains differed in their response to iron with regard to yields of extracellular proteases.

P. aeruginosa produces at least 3 separate proteases: protease I, II (elastase) and III (20, 31). The results obtained when total extracellular protease yields were quantified (Table 3A) might be due to different levels of sensitivity of the individual proteases to the iron concentration of the growth medium. Therefore, we examined the effect of the iron concentration of the growth medium on the yields of one of the individual proteases, elastase. We studied strains WR-9 and WR-28 because they represented 2 strains of P. aeruginosa whose total extracellular protease yields were markedly decreased when grown in high iron medium and strain WR-27 because it was the only strain tested whose yield of total extracellular protease increased when grown in high iron medium (Table 3A). While not identical to the effect seen on total protease yields, the yields of elastase by strains WR-9 and WR-28 were markedly decreased in cultures grown in high iron medium. On the other hand, the yield of elastase by strain WR-27 was increased in the high iron medium as was the yield of total protease (Tables 3A and 3B).

Hemagglutinin yields in high and low iron medium. During the course of developing a reversed passive hemagglutinin assay for toxin A we noticed that many strains of P. aeruginosa produced an extracellular factor that caused the agglutination of formalinized sheep red blood cells (SRBC). We termed this factor a hemagglutinin (HA). To our knowledge, the identification of an extracellular P. aeruginosa

product that has hemagglutinin activity has not previously been reported. The HA as it was found in crude supernatant fluid was non-dialysable, heat stable (100° C for 15 min), trypsin and pronase resistant.

The effect of increasing the concentration of iron in the growth medium on the yields of the HA was investigated. Five of the seven strains showed a dramatic decrease in HA yields in medium supplemented with 5 μg iron/ml (Table 4). With these five strains the relationship between iron and HA yields resembled those seen with toxin A (Table 2). However, strain PA-103 produced very small amounts of HA and the yields were not altered by increasing the iron concentration of the growth medium. With strain WR-28, HA was not detectable in the low iron medium but was detectable when the organism was grown in the high iron medium (Table 4). Thus, as was found with total extracellular proteases the effect of iron on HA yields was strain dependent.

We have used extensively dialysed supernatants in these studies to remove any effects that iron might have on the activities of preformed toxin A, proteases or HA. Furthermore, we have previously shown that iron at concentrations used in this study had no effect on crude or pure toxin A activity (1). We have also found that 5 μg iron/ml had no effect on the activity of preformed P. aeruginosa proteases or HA (data not shown). Thus the data (Tables 2, 3, 4) on the effect of iron on the yields of toxin A, protease or HA cannot be accounted for by the effect of iron on the activities of these extracellular products.

The effect of iron on yields of total extracellular protein.

Each of the seven strains showed decreased yields of one or more extracellular products besides toxin A when the concentration of iron in the growth medium was increased. Therefore, we examined the effect of the iron content of the medium on the yield of total extracellular protein. The amount of protein was expressed as a function of bacterial growth (μg extracellular protein/ml/OD_{540} unit) to compensate for the differences in growth between the cultures grown in high and low iron medium (Table 2). The amount of extracellular protein/ml/OD_{540} unit was dramatically decreased in the high iron medium for all strains of P. aeruginosa examined (Table 5). The magnitude of the decrease was most apparent with strain PA-103. With PA-103 the yield of total extracellular protein decreased 87% when the iron content of the medium was increased from 0.05 μg/ml to 5.0 μg/ml. Yields of extracellular protein decreased from 31 to 67% with the other strains (Table 5). Results similar to those shown have been found using viable bacterial counts (colony forming units) as a measure of bacterial growth instead of optical density at 540 nm (data not shown). Similar to results shown earlier (Table 2) the yields of toxin A were markedly decreased when the iron content of the medium was increased (Table 5). Comparisons between low iron and high iron growth medium showed the percent decrease in yields of toxin A was greater for each strain tested than was the percent decrease in extracellular protein. However, the decrease in the total extracellular protein was greater than could be accounted for by the decrease in toxin A yields (Tables 2

and 5). When PA-103 was grown in high iron medium the total protein was reduced by 87% as compared to total protein in the low iron medium. However, the maximum amount of toxin A, produced under ideal conditions (low iron) represented only 5% of the total extracellular protein (Table 5). This data is consistent with our finding that the yields of other extracellular products besides toxin A are decreased as the iron concentration of the growth medium increases.

Effect of varying concentrations of iron in the medium on growth and yields of extracellular products by P. aeruginosa WR-9. The yields of toxin A, total proteases and HA were all markedly decreased in culture supernatants of strain WR-9 when the iron concentration of growth medium was increased from 0.05 to 5 µg/ml (Tables 2, 3, 4, 5). To further evaluate the relationship between the iron concentration and yields of these products we examined their yields in growth medium containing various concentrations of iron (Fig. 2). The final yield of bacteria increased as the concentration of iron in the medium increased until at concentrations greater than 0.5 µg/ml iron was no longer limiting bacteria growth (Fig. 2). The yields of all three extracellular products tested (toxin A, total proteases and HA) were dramatically decreased as the iron concentration of the medium was increased from 0.05 to 0.2 µg/ml (Fig. 2). The inhibitory effect of iron began to level off at concentrations greater than 0.5 µg/ml but small amounts of these extracellular products could still be detected

in cultures containing 5 µg iron/ml (Fig. 2). The three curves showing the effect of iron on the yields of these products were similar (Fig. 2).

The effect of iron on the rates of release of extracellular products by P. aeruginosa strain WR-9. We determined the rates at which toxin A, total proteases and HA were released into the supernatant fluid and the effect of iron on these rates. The yield of bacteria was greater in the high iron medium than in the low iron medium and began to level off at about 20 h (Fig. 3A). Toxin A in the low iron medium was first detectable at about 10 h and continued to increase through 22 h. In contrast, the yield of toxin A was dramatically decreased in the high iron medium (Fig. 3A). Toxin A in the high iron medium was first detectable at 16 h and even at 22 h the levels of toxin A were barely detectable in our transferase assay (Fig. 3A). Proteolytic activity in the low iron medium was first detectable at 10 h and continued to increase through 20 h (Fig. 3B). In the high iron medium the yield of protease was markedly depressed and was first detectable at 12 h (Fig. 3B). The rate of HA release followed the same basic pattern as toxin A and protease release. HA (8 HA units/50 µl) was first detectable at 12 h in the low iron medium and increased through 18 h. By 18 h the HA reached a titer of 640 HA units/ml and did not increase any further. The maximum yields of HA in the high iron medium was 16 HA units/50 µl (Fig. 3B).

We also examined the rate of release of total extracellular protein using strain WR-9. In agreement with our preceding data (Table 5)

the amount of extracellular protein/ml/OD_{540} unit was decreased when the organism was grown in the high iron medium. This decrease in extracellular protein yields in high iron medium was evident at all times tested (13, 15 and 22 h). Most of the extracellular protein was released into the supernatant fluid between 12 and 22 h regardless of whether the cultures were grown in high or low iron medium (data not shown).

Discussion

Previously, we reported that increasing the iron concentration of the growth medium decreased the yields of toxin A using *P. aeruginosa* strain PA-103 (1). That study was limited to only one strain (PA-103) of *P. aeruginosa* and only one extracellular product (toxin A) was examined. The present study utilized seven different strains of *P. aeruginosa*. We examined the effect of the iron concentration of the medium on bacterial growth, yields of four extracellular products (toxin A, total proteases, elastase and HA) and total extracellular protein. To our knowledge the identification of a *P. aeruginosa* factor with hemagglutinating activity has not previously been described. The biological significance of the HA is not known at this time.

The final yield of bacteria (22 h cultures) in the high iron medium was about twice that found in the low iron medium for all seven strains of *P. aeruginosa* tested (Table 2). In contrast, yields of the individual extracellular products generally decreased with increasing concentrations of iron in the growth medium (Table 2, 3, 4). The

effect of increasing the concentration of iron in the medium on toxin A yields was strain independent. The magnitude of inhibition on toxin A yields in the presence of increasing concentration of iron was similar in all seven strains (Table 2), and consistent with our previous report (1). On the other hand, the effect of increasing the iron concentration of the growth medium on yields of total proteases, elastase and HA was strain dependent (Tables 3 and 4). Increasing the iron concentration of the growth medium resulted in decreased extracellular yields of total proteases in 6 of 7 strains, elastase in 2 of 3 strains and HA in 5 of 7 strains. Yields of total proteases and elastase were increased slightly in cultures of strain WR-27 and yields of HA increased in cultures of strain WR-28 and remained unaltered (but very low) with strain PA-103 when the iron concentration of the medium was increased from 0.05 to 5 μg/ml (Table 4). These results suggest that in some strains of P. aeruginosa (i.e. WR-27 and WR-28) proteases and/or HA yields are regulated by iron independently from its regulation of toxin A.

The amount of total extracellular protein, when normalized for bacterial growth, was markedly decreased in all strains tested by increasing the concentration of iron in the growth medium (Table 5). The yields of total extracellular protein could not be accounted for simply by the decreased toxin A yields (Table 5). This is consistent with our finding that the yields of other P. aeruginosa extracellular proteins (total protease and elastase) were generally decreased as the concentration of iron in the growth medium was increased. Furthermore, this effect was not restricted to extracellular proteins but includes

other extracellular products. Several investigators have shown that iron inhibits the yields of the fluorescein and pyocyanine pigments produced by P. aeruginosa (3, 10, 14, 28). In the current study, we observed that yields of HA were generally reduced by increased concentrations of iron in the growth medium (Table 4). Based on our observations with toxin A, total extracellular proteases, elastase, HA and total extracellular protein (Tables 2, 3, 4 and 5) and those reported earlier for P. aeruginosa pigments, we expect that the yields of still other extracellular products will be similarly influenced by the iron concentration of the medium.

When grown in high iron medium, four of the seven P. aeruginosa strains that we examined (WR 4, 9, 35, 56) showed significant decreases in the yields of three extracellular products examined (toxin A, total proteases and HA). A more extensive examination employing strain WR-9 indicated that in the presence of increasing amounts of iron in the medium, the yields of these three products were decreased in a similar manner (Fig. 2). As little as 0.5 µg iron/ml decreased the yields of toxin A, proteases and HA by at least 80% as compared to the yields of these products in medium containing only 0.05 µg iron/ml. The rates of release of these extracellular products were also similar at a given iron concentration (Fig. 3). Toxin A, proteases and HA were first detectable at about 10 h in the low iron medium and followed approximately the same rates of release through 18 h. In contrast, when WR-9 was grown in the high iron medium, the rates of release of these products were markedly decreased (Fig. 3). These

results suggest that in strain WR-9 iron regulates the release of toxin A, proteases and HA either by some common mechanism or through equally sensitive independent mechanisms.

Yields of diphtheria, Shigella dysenteriae type I and Pseudomonas A toxins have been shown to decrease as the iron concentration of the growth medium increases (1, 5, 19, 23, 26, 29). This study extends this effect of iron to include additional extracellular products of P. aeruginosa. The mechanism(s) by which iron exerts this control is unknown.

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Table 1. Characterization of *P. aeruginosa* strains

STRAIN	SOURCE	SEROTYPE	PYOCIN TYPE	TOXIN A	PROTEASE	ELASTASE	HEMAGGLUTININ
PA 103	Sputum	2	611 131	+	+	-	+
WR 4	Perineal ulcer	NR[a]	587 688	+	+	+	+
WR 9	Wound	2	617 161	+	+	+	+
WR 27	Blood	1	621 611	+	+	+	+
WR 28	Sputum	6	211 216	+	+	+	+
WR 35	Wound	5	111 214	+	+	+	+
WR 56	Sputum	3 & 7	113 216	+	+	+	+

[a]NR; no reaction (agglutination) with any of the 7 typing sera.

Table 2. Effect of iron on bacterial growth and toxin A yields in cultures of P. aeruginosa

	Bacterial growth (OD_{540})		µg toxin A/ml	
Strain	Low (0.05 µg/ml) iron	High (5.0 µg/ml) iron	Low (0.05 µg/ml) iron	High (5.0 µg/ml) iron
PA-103	8.1	18.7	25.5	2.9
WR-4	6.8	14.4	2.5	ND[a]
WR-9	8.3	16.5	4.5	0.05
WR-27	7.4	17.7	1.3	ND
WR-28	8.9	18.8	3.2	0.07
WR-35	6.3	14.7	1.5	ND
WR-56	6.9	15.5	0.4	ND

[a]ND, not detectable

Table 3. Effect of iron on protease yields in cultures of *P. aeruginosa*

A. Total extracellular proteases

Proteolytic units/ml

Strain	Low (0.05 µg/ml) iron	High (5.0 µg/ml) iron	% decrease
PA-103	0.3	0.02	93
WR-4	13.3	10.1	34
WR-9	7.8	3.0	62
WR-27	9.2	12.1	0
WR-28	2.3	0.1	96
WR-35	11.6	6.1	47
WR-56	12.7	9.6	24

B. Extracellular elastase

Elastolytic units/ml

Strain	Low (0.05 µg/ml) iron	High (5.0 µg/ml) iron	% decrease
WR-9	0.28	0.04	86
WR-27	0.31	0.37	0
WR-28	0.36	0.18	50

Table 4. Effect of iron on the yields of hemagglutinin in cultures of *P. aeruginosa*

Strain	Hemagglutinin titre	
	Low (0.05 µg/ml) iron	High (5.0 µg/ml) iron
PA-103	2	2
WR-4	64	0
WR-9	256	8
WR-27	64	8
WR-28	0	16
WR-35	512	64
WR-56	16	0

Table 5. Effect of iron on yields of extracellular protein and toxin A in cultures of *P. aeruginosa*

	μg extracellular protein/ml/OD_{540} unit		μg toxin A/ml/OD_{540} unit	
Strain	Low (0.05 μg/ml) iron	High (5.0 μg/ml) iron	Low (0.05 μg/ml) iron	High (5.0 μg/ml) iron
PA-103	58.4	7.6 (87%)[a]	3.1	0.16 (95%)[a]
WR-4	42.7	29.2 (32%)	0.37	≤0.003 (≥99%)
WR-9	40.4	15.4 (62%)	0.54	0.003 (99%)
WR-27	29.3	16.0 (45%)	0.18	≤0.003 (≥98%)
WR-28	27.0	15.6 (42%)	0.36	0.004 (99%)
WR-35	44.4	30.6 (31%)	0.24	≤0.003 (≥98%)
WR-56	55.1	18.1 (67%)	0.06	≤0.003 (≥95%)

[a]Numbers in parenthesis represent percent decrease in yields of total extracellular protein or toxin A in the high iron medium as compared to low iron medium.

Figure 1. ADPR Transferase assay: standard curves obtained with pure toxin A. A) standard curve obtained using a 5 min assay system. B) standard curve obtained using a 30 min assay system.

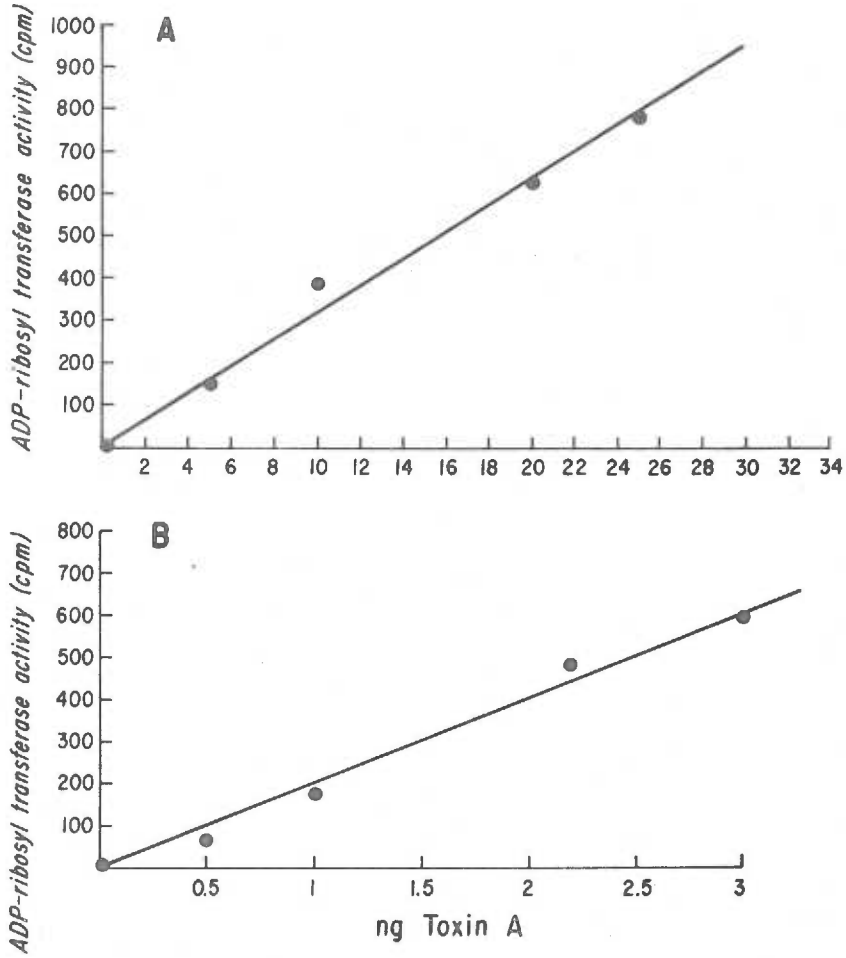

A
ADP-ribosyl transferase activity (cpm)
1000
900
800
700
600
500
400
300
200
100
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34
B
ADP-ribosyl transferase activity (cpm)
800
700
600
500
400
300
200
100
0.5 1 1.5 2 2.5 3
ng Toxin A

Figure 2. Effect of increasing concentrations of iron on the yields of bacterial growth, toxin A, proteases and hemagglutinin in cultures of P. aeruginosa WR-9. Yields of the extracellular products in the low iron (0.05 µg/ml) medium were considered 100%.

Symbols: (○) bacterial growth; (●) toxin A yields; (■) protease yields; (▲) HA yields.

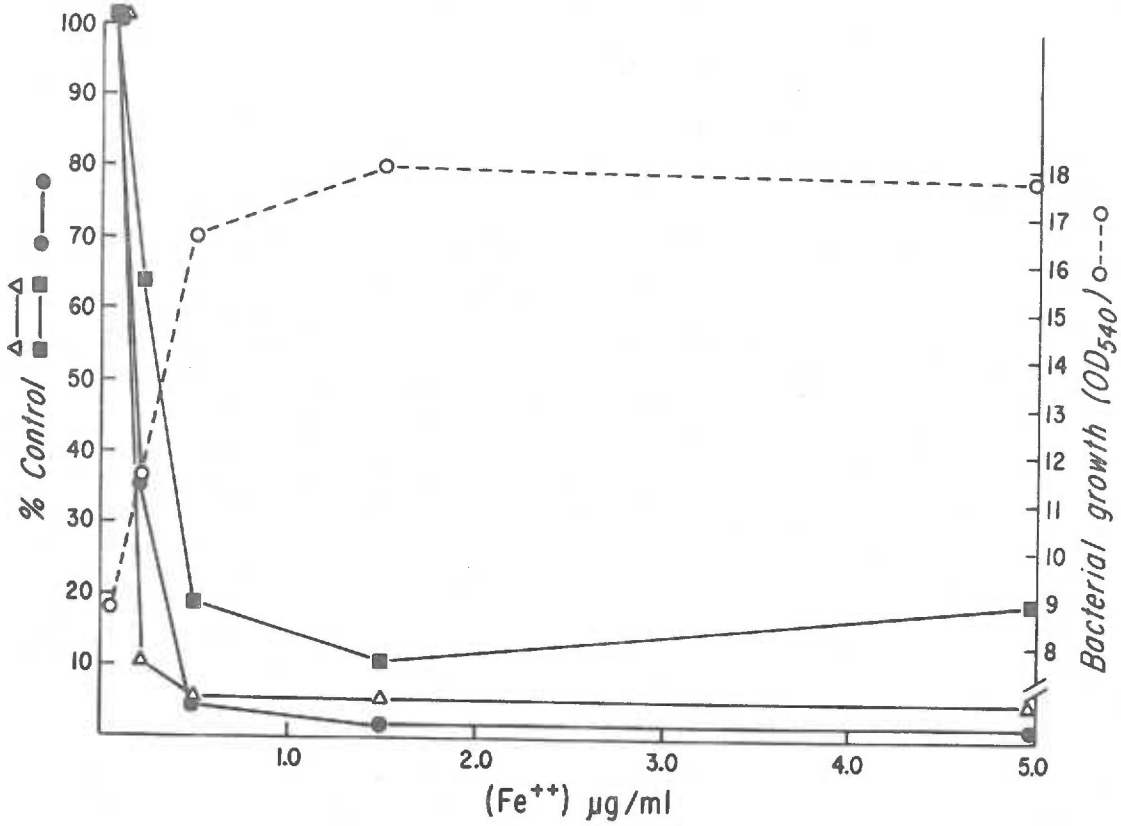
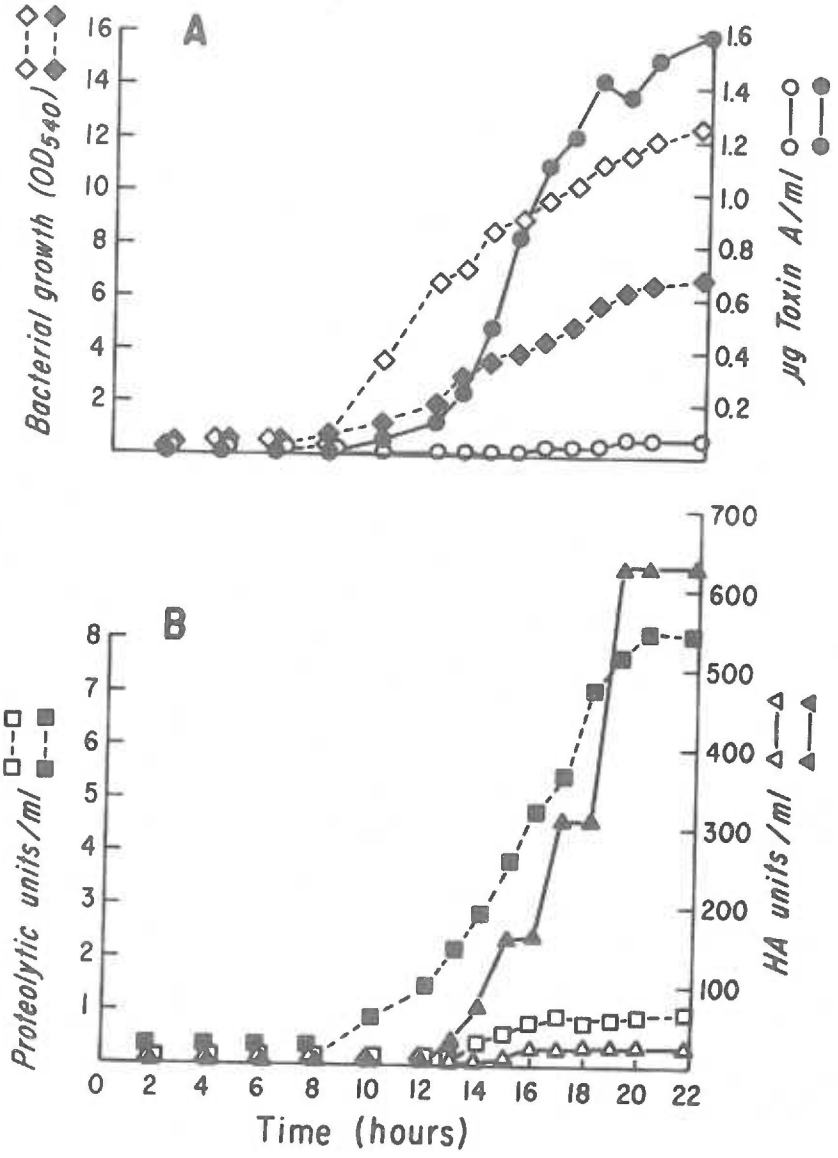

100
90
80
70
60
50
40
30
20
10
% Control
18
17
16
15
14
13
12
11
10
9
8
Bacterial growth (OD540)
1.0
2.0
3.0
4.0
5.0
(Fe++) µg/ml

Figure 3. Effect of low (0.05 µg/ml) and high (5.0 µg/ml) iron concentrations on A) bacterial growth and toxin A release and B) protease and HA release in cultures of P. aeruginosa WR-9. Symbols: (◇, ◆) bacterial growth; (○, ●) toxin A; (□, ■) protease; (△, ▲) HA. The open symbols (◇, ○, □, △) represent yields in high iron medium, the closed symbols (◆, ●, ■, ▲) in low iron medium.


A
Bacterial growth (OD_{540})
µg Toxin A/ml
16
14
12
10
8
6
4
2
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
B
Proteolytic units/ml
HA units/ml
8
7
6
5
4
3
2
1
700
600
500
400
300
200
100
0 2 4 6 8 10 12 14 16 18 20 22
Time (hours)

Paper 4.

Pseudomonas aeruginosa exoenzyme S: an adenosine diphosphate ribosyl transferase distinct from toxin A.

Abstract

Pseudomonas aeruginosa exoenzyme S is an adenosine diphosphate ribosyl transferase distinct from *Pseudomonas* toxin A. Exoenzyme S catalyzes the transfer of radioactivity from all portions of radiolabeled NAD^+ except nicotinamide. Digestion of the radiolabeled product(s) formed in the presence of [adenine-^{14}C]NAD^+ and exoenzyme S with snake venom phosphodiesterase yields only AMP, suggesting that ADP-ribose is present as monomers and not as poly(ADP-ribose). Exoenzyme S does not catalyse the transfer of ADP-ribose from NAD^+ to elongation factor-2, as do toxin A and diphtheria toxin, but to one or more other proteins present in partially purified preparations of elongation factor-1 and in crude extracts of wheat germ or rabbit reticulocytes. The ADP-ribosyl transferase activity of exoenzyme S is distinct from toxin A by several tests: it is not neutralized by toxin A antibody, is destroyed rather than potentiated by pretreatment with urea and is more heat-stable. These latter observations and the substrate specificity suggest that exoenzyme S is different from any previously described prokaryotic ADP-ribosyl transferase.

Introduction

Diphtheria toxin and *Pseudomonas* toxin A inhibit protein synthesis in eukaryotic cells by catalyzing the transfer of the ADP-ribose (ADP-Rib) moiety of NAD^+ to elongation factor-2 (EF-2) (4, 9, 10). The only eukaryotic protein known to be modified by these two toxins

is EF-2, and all existing information supports the conclusion that the ADP-ribosylation of EF-2 is responsible for the lethality of these two toxins (4, 11). Diphtheria toxin is encoded by a phage gene (4, 24) but the location of the structural gene for Pseudomonas toxin A is presently unknown. Approximately 90% of all isolates of Pseudomonas aeruginosa tested produce toxin A (2, 21).

In this report we describe a new ADP-ribosyl transferase (exoenzyme S) that is present in the culture supernatant fluid of a strain of P. aeruginosa (Ps 388). Ps 388 was consistently negative in an immune precipitation assay (2) using specific toxin A antibody. In the presence of limiting amounts of EF-2, exoenzyme S catalyzed the transfer of far more ADP-Rib from NAD^+ than could be accounted for by the production of ADP-ribosylated EF-2. Data are presented to show that exoenzyme S, unlike diphtheria or Pseudomonas A toxins, does not modify EF-2 but some other eukaryotic protein(s). ADP-Rib appears to be present in the modified protein(s) as monomeric units rather than as poly(ADP-Rib). We also show that exoenzyme S is distinct from Pseudomonas toxin A by several other tests. It is not neutralized by toxin A antibody, is destroyed rather than potentiated by pretreatment with urea and is more heat-stable.

Materials and Methods

Exoenzyme S and toxins. Ps 388, kindly provided by Dr. B. Minshew (Seattle, Wa.) was grown in a liquid medium adjusted to pH 7.0 consisting of the dialysate from trypticase soy broth (Baltimore

Biological Lab.) (16) supplemented with 0.1 M monosodium glutamate, 1% glycerol and 0.01 M nitrilotriacetic acid (Sigma Chem. Co.). A 25 ml amount of this medium in a 500 ml Erlenmeyer flask was inoculated with an overnight culture of Ps 388 to an initial cell density of approximately 5×10^7 cells/ml. The culture was incubated at 32° C on a reciprocating shaker (200 linear excursions/min) (Lab-line Inst.) for 22 h. The culture supernatant fluid was obtained by centrifugation at 10,000 x g for 20 min at 4° C, was filter-sterilized and stored at -70° C in small aliquots. Crude exoenzyme S, present in the culture supernatant fluid prepared and stored in this manner, retains enzymatic activity for several months.

P. aeruginosa (PA-103) (16) was used as a source of toxin A which was produced and purified as described previously (12). The purified toxin A had a mouse LD_{50} of 0.2 µg/22 g mouse when injected intraperitoneally. Fragment A was obtained by treating purified diphtheria toxin with trypsin and dithiothreitol (5). The fragment A was then chromatographed on Sephadex G-100 with 1 mM EDTA, 1 mM dithiothreitol and 50 mM Tris-HCl pH 7.8.

Enzymatic activity. Crude extracts containing aminoacyl transferase factors were prepared from wheat germ as described by Chung and Collier (3). ADP-ribosyl transferase activity was measured by the incorporation of radioactivity from [adenine-^{14}C]NAD^+ into trichloroacetic acid-precipitable material in the presence of crude wheat germ extracts as previously described (12). Unless otherwise noted, the reaction was performed at 25° C for 5 min in 0.1 ml of

50 mM Tris-HCl (pH 7.0); 1 mM EDTA; 50 mM dithiotreitol; 0.12 mM [adenine-^{14}C]NAD$^+$ (10.6 mCi/mmole) (Amersham/Searle); wheat germ extract containing 150-160 µg proteins and various amounts of exoenzyme S, diphtheria toxin fragment A or Pseudomonas toxin A. The reaction was stopped by the addition of 0.1 ml 10% trichloroacetic acid, the precipitates were collected, washed and counted as previously described (12). The enzymatic activity of Pseudomonas toxin A was potentiated by preincubating the toxin A in 4 M urea and 1% dithiothreitol for 15 min at 25° C (25). Where noted [nicotinamide-^{14}C]NAD$^+$ (59 mCi/mmole) (Amersham/Searle) or [Rib(NMN)-^{14}C]-NAD$^+$ (75 mCi/mmole) prepared as previously described (13) was substituted for the [adenine-^{14}C]NAD$^+$. The following proteins were used in experiments designed to elucidate the substrate specificity of exoenzyme S: crude extract of rabbit reticulocytes prepared as described by Allen and Schweet (1) as modified by Collier and Kandel (5), purified EF-2 (17) and elongation factor-1 (EF-1) (14) from rabbit reticulocytes, (kindly provided by Dr. W. Merrick, NIH), rat liver EF-1 partially purified as previously described (6), poly L-lysine (Sigma), bovine serum albumin (BSA) (Sigma), egg white lysozyme (Calbiochem). RNase and DNase (Worthington Biochem.).

Enzyme neutralization. Specific toxin A antiserum was produced in rabbits as previously described (2). Enzyme-inactivation by antibody was determined by assaying the ADP-ribosyl transferase activity (5 min incubation at 25° C) after preincubation of 0.1 µg toxin A or 0.15 µg protein in the crude exoenzyme S with antiserum or normal

rabbit serum for 5 min at 37° C as previously described (10). The antiserum was diluted in saline containing 0.1 mg of bovine serum albumin (BSA) per ml.

Analysis of the reaction product. Radiolabeled products formed in the ADP-ribosyl transferase assays were electrophoresed in sodium dodecyl sulfate (SDS)/polyacrylamide gels by a previously described (25) modification of the method of Weber and Osborn (26). Gel slices (1 mm thick) were incubated 12 h at 25° C in 1 ml buffer (0.1 M Tris-HCl pH 7.5; 10 mM EDTA and 0.1 mg BSA). The eluted protein was precipitated with trichloroacetic acid (5%), collected on Millipore filters and washed. Radioactivity was measured as previously described (10).

In order to determine if ADP-Rib was present in the acceptor protein(s) as monomeric units or as poly(ADP-Rib), a 0.3 ml reaction mixture containing 50 mM Tris-HCl (pH 7.0), 0.03 mM EDTA, 50 mM dithiothreitol, 0.14 mM [adenine-^{14}C]NAD^{+} (26 mCi/mmole) rat liver EF-1 (60 µg protein) and 50 µl exoenzyme S was incubated for 150 min at 25° C. At 60, 90, 120 and 150 min, 10 µl aliquots were removed and trichloroacetic acid-precipitable radioactivity was determined. No futher incorporation of radioactivity occurred after 120 min. The reaction mixture remaining (260 µl) was chromatographed on a column of Sephadex G-25 (bed volume, 4.0 ml) in 0.2 M Tris·HCl (pH 7.0) to separate the labeled product(s) from unreacted [^{14}C]NAD^{+}. Fractions containing high-molecular weight radioactive material were pooled (total volume, 0.56 ml) and an aliquot of 250 µl was incubated for 60 min with 240 µg snake venom phosphodiesterase (Sigma) in a total

volume of 0.5 ml containing 0.1 M Tris-HCl (pH 7.0) and 20 mM $MgCl_2$. The products of this digestion were chromatographed on Sephadex G-25 (equilibrated and eluted with water), and the included low-molecular weight radioactive material was concentrated by lyophilization and resuspended in water. The digested products were then chromatographed on thin layer polyethyleneimine-cellulose plates (J.T. Baker) employing 0.3 M lithium chloride as the solvent (23). AMP, ADP-Rib (Calbiochem) and NAD^+ (Sigma) were cochromatographed as markers. The chromatograph was cut into small pieces and eluted with 0.3 ml 1.6 M lithium chloride. After addition of 5 ml scintillation fluid (16.5 g diphenyloxazole dissolved in 2 liter toluene and 1 liter Triton X-100) radioactivity of the samples was determined in a Nuclear Chicago scintillation counter.

Results

The culture supernatant fluid of P. aeruginosa strain 388 (Ps 388) contained activity which, like Pseudomonas toxin A (12), transferred radioactivity from [adenine-^{14}C]NAD^+ into acid-insoluble material in the presence of crude wheat germ extracts. Nevertheless, Ps 388 was consistently negative in immune precipitation assays (2) with rabbit toxin A antiserum. Similarly, preparations of culture supernatant fluid from Ps 388 failed to react with toxin A antiserum in agar gel diffusion assays, even when the preparations were concentrated 50 fold and a wide range of antiserum dilutions was tested. We then compared the ability of the toxin A antiserum to neutralize the ADP-ribosyl

transferase activity of toxin A and the activity present in the culture supernatant fluid of Ps 388 (exoenzyme S). As shown in Fig. 1, the toxin A antiserum neutralized 80-100% of the enzymatic activity of pure toxin A at dilutions of 1:16 or less. Similar results were obtained with crude toxin A preparations or a purified enzymatically active peptide (25) derived from toxin A (data not shown). In contrast, even at the highest concentration of toxin A antiserum used (dilution 1:2) the enzymatic activity of exoenzyme S was not decreased (Fig. 1).

To determine which portions of NAD^+ are transferred by exoenzyme S, the enzyme was incubated with wheat germ extracts and NAD^+ radioactively labeled at different positions. All portions of NAD^+ except nicotinamide were incorporated into trichloroacetic acid-insoluble reaction product(s) in the presence of exoenzyme S or toxin A (Table 1). Thus exoenzyme S is an ADP-ribosyl transferase.

Pseudomonas toxin A is produced as a toxic proenzyme that is virtually devoid of ADP-ribosyl transferase activity (3, 15, 25). The transferase activity of toxin A is expressed when the molecule is cleaved by proteolysis to yield an enzymatically active fragment (3, 25) or when it is denatured and reduced (15, 25). In contrast, we found that treatment of exoenzyme S with 4 M urea markedly reduced its enzymatic activity. The presence of dithiothreitol slightly modified the effect of urea on exoenzyme S activity (Table 2).

The enzymatic activity of toxin A and that of the enzymatically active peptide derived from toxin A was heat sensitive (3, 10 and

Table 3). The enzymatic activity of exoenzyme S was relatively heat stable. As shown in Table 3 at least 90% of the enzymatic activity of either crude or purified toxin A was destroyed by incubation at 100° C for 2 min whereas only 30% of the activity of exoenzyme S was lost after 10 min at 100° C. The data in Fig. 1 and Tables 2 and 3 suggest that exoenzyme S and toxin A are structurally different.

The data in Table 4 show that in the presence of excess NAD^+ and limiting amounts of wheat germ extract, exoenzyme S catalyzed the transfer of far more ADP-Rib than could be accounted for by the formation of an ADP-ribosylated EF-2 product such as has been previously described (4, 9, 10, 11). When excess toxin A or fragment A was incubated with limiting amounts of wheat germ extract and excess NAD^+, approximately 32 pmoles ADP-Rib was transferred from [adenine-^{14}C]NAD^+ to acid-insoluble material. Doubling the amount of toxin A or fragment A did not increase the amount of ADP-Rib transferred. Under the same conditions, 10 µl of exoenzyme S catalyzed the transfer of 3670 pmoles of ADP-Rib.

An investigation of the product formed in the presence of exoenzyme S revealed it to be stable to treatment with DNase or RNase but digestible with trypsin or pronase. When this radiolabeled product was subjected to electrophoresis on SDS polyacrylamide gels, the acid-precipitable material obtained after elution of the individual gel slices indicated the product was heterogenous in size (Fig. 2). No significant amount of radioactivity was associated with material larger than 50,000 daltons. In agreement with previous reports (10,

11) the product formed in the presence of toxin A was homogenous and had a molecular weight of 100,000 (the known molecular weight of EF-2) (Fig. 2).

These data (Table 4 and Fig. 2) suggested that EF-2 was not the acceptor for ADP-Rib in the reaction catalyzed by exoenzyme S. This was confirmed in experiments utilizing highly purified reticulocyte EF-2. As shown in Table 5, exoenzyme S failed to transfer [adenine-^{14}C]ADP-Rib from [adenine-^{14}C]NAD^+ to pure EF-2. Under the same conditions 0.2 µg toxin A catalyzed the transfer of 2050 pmoles ADP-Rib to this preparation of EF-2 (data not shown).

A variety of other proteins were tested for acceptor activity in the reaction catalyzed by exoenzyme S (Table 5). Poly(L-lysine), bovine serum albumin, egg white lysozyme and RNase were inactive. The two EF-1 preparations tested contained acceptor activity. The rat liver EF-1 and reticulocyte EF-1 preparations, purified by different methods, were reported to be 50 and 95% pure, respectively (6, 14). Thus the acceptor(s) could well be contaminating proteins and not EF-1. However, a substantial amount of the radioactive product in the wheat germ extract was associated with material of about 50,000 molecular weight (Fig. 2), a result consistent with ADP-ribosylation of EF-1. The smaller labeled material (Fig. 2) might have resulted from proteolysis since the exoenzyme S preparation contained contaminating proteases.

Previously described prokaryotic ADP-ribosyl transferases have been shown to transfer single molecules of ADP-Rib from NAD^+

directly to proteins and not to each other in a repetitive fashion to yield poly(ADP-Rib) (8). In order to determine if the protein(s) modified by exoenzyme S contained monomers or polymers of ADP-Rib the product(s) formed in the presence of exoenzyme S, [adenine-^{14}C] NAD^+ and rat liver EF-1 were synthesized and treated as described in Materials and Methods. Eighty-two percent (2.0 nmoles) of the acid-precipitable radioactivity in the original reaction mixture was recovered from Sephadex G-25 as high-molecular weight material. Following incubation with venom phosphodiesterase only 62% of the applied radioactive material was recovered, but all the recovered activity was in low-molecular weight fractions. Ninety-three percent of this low-molecular weight material chromatographed with AMP on polyethyleneimine-cellulose plates. No radioactivity migrated with marker ADP-Rib [which co-migrates in the system used with isoADP-Rib, the product of venom phosphodiesterase action on poly(ADP-Rib)] (8). When the product formed with wheat germ extract was analyzed similarly, the only radioactive product recovered after snake venom phosphodiesterase digestion was AMP. This strongly suggests that ADP-Rib is present on the acceptor(s) in both wheat germ extracts and in the rat liver EF-1 preparation as monomeric units, rather than as poly(ADP-Rib).

Discussion

Five prokaryotic ADP-ribosyl transferases have been described: diphtheria toxin (4, 9); Pseudomonas toxin A (10, 11); T4 phage "alteration" enzyme (27); T4 phage "modification" enzyme (7) and an N4

phage enzyme (20). Existing evidence indicates these enzymes play major roles in infection (i.e., diphtheria toxin) or in the regulation of various cellular activities (i.e., T4 modification enzyme) (4, 8). In addition, preliminary evidence has been presented that cholera toxin may be an ADP-ribosyl transferase (18).

The present results demonstrate the existence of a sixth prokaryotic ADP-ribosyl transferase, exoenzyme S, which is produced by some strains of P. aeruginosa. Exoenzyme S differs immunologically from Pseudomonas toxin A, is relatively heat stable compared to toxin A, and is partially inactivated by conditions that potentiated the enzymatic activity of toxin A (Fig. 1, Tables 2 and 3). Exoenzyme S catalyzed the transfer of ADP-Rib from NAD^+ to protein(s) present in wheat germ extracts, rabbit reticulocyte extracts, and partially purified EF-1 preparations (Tables 4 and 5). Elongation factor-2 failed to serve as an acceptor in the reaction catalyzed by exoenzyme S, indicating a substrate specificity different from that of Pseudomonas toxin A and diphtheria toxin. Furthermore, lysozyme, which serves as an acceptor in the in vitro reaction catalyzed by the T4 phage alteration enzyme (27) and the N4 virion associated enzyme (20), was not ADP-ribosylated in the presence of exoenzyme S. The T4 modification enzyme reportedly does not ADP-ribosylate proteins present in rabbit reticulocyte extracts (7) as did exoenzyme S (Table 5). Thus exoenzyme S differs in its substrate specificity from previously described prokaryotic ADP-ribosyl transferases.

Our results raised the possibility that exoenzyme S ADP-ribosylates EF-1. As seen in Table 5, in the presence of exoenzyme S [^{14}C]ADP-Rib was transferred from [adenine-^{14}C]NAD^{+} to trichloroacetic acid-insoluble material when EF-1 preparations were used as the substrate. The rat liver EF-1 preparation was approximately 50% pure and contained 4 or 5 proteins other than EF-1 (6). The reticulocyte EF-1 preparation was approximately 95% pure (14). These proteins were purified by different procedures (6, 14). Considerably more ADP-Rib/μg protein was transferred to the rat liver EF-1 preparation than the reticulocyte EF-1 preparation. Thus, if exoenzyme S is ADP-ribosylating EF-1, then either EF-1 from different sources differs in the extent to which it can be modified by exoenzyme S or it may somehow be altered during purification. A third, perhaps more likely possibility, is that some other protein(s) contaminating the EF-1 preparations is being ADP-ribosylated by exoenzyme S and this is in a smaller concentration in the more highly purified reticulocyte EF-1 preparations than in the rat liver EF-1 preparation. Purification of exoenzyme S to remove proteolytic contaminants, together with the use of recently developed methods for purifying catalytically active EF-1 and EF-1 α and β factors (13), should provide the necessary reagents to distinguish among these possibilities.

The role of diphtheria toxin in C. diphtheriae infections is clear (4). There is increasing evidence that toxin A is also important in the pathogenesis of disease caused by P. aeruginosa (2, 11, 19, 21). P. aeruginosa strain 388 produces exoenzyme S but no

detectable amounts of toxin A. More recently we have identified other strains of P. aeruginosa that produce exoenzyme S and several that produce both toxin A and exoenzyme S. The substrate(s) modified by exoenzyme S, the consequences of the modification, and the role of exoenzyme S in the pathogenesis of P. aeruginosa infections are currently being investigated.

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Table 1. Incorporation of label from NAD^+ preparations into protein by exoenzyme S

NAD^+ used	Radioactivity incorporated, CPM	
	Minus enzyme	Plus enzyme
[Adenine-^{14}C]NAD^+	180	26,000
[Rib(NMN)-^{14}C]NAD^+	220	3,560
[Nicotinamide-^{14}C]NAD^+	110	118

[Adenine-^{14}C]NAD^+, 5.0 μM (266 mCi/mmole), [Rib(NMN)-^{14}C]NAD^+, 3.0 μM (75 mCi/mmole) or [nicotinamide-^{14}C]NAD^+, 5.0 μM (59 mCi/mmole) were added to the reaction mixtures containing 10 μl H_2O or 10 μl exoenzyme S, 150 μg wheat germ extract and reaction buffer. Incubation was for 5 min.

Table 2. Effect of urea and dithiothreitol on ADP-ribosyl transferase activity of exoenzyme S and Pseudomonas toxin A

Treatment	ADP-Rib incorporated, pmoles	
	Toxin A	Exoenzyme S
H_2O	0.72	1200
1% Dithiothreitol	0.67	1250
4M Urea	10.9	760
4M Urea + Dithiothreitol	26.1	960

Pseudomonas toxin A (.02 µg) or exoenzyme S (5 µl) was mixed with 5 µl of the solution to be tested and incubated at 25° C for 15 min, then immediately assayed for ADP-ribosyl transferase activity as described in Materials and Methods. Incubation was for 5 min.

Table 3. Comparison of the thermal stability of *Pseudomomas* toxin A and exoenzyme S

	% Unheated enzymatic activity		
Time (min)	Exoenzyme S	Crude toxin A	Purified toxin A
0	100	100	100
2	111	6	10
5	97	3	6
10	71	1	4
20	11	3	2
30	0.3	1	1

Samples (exoenzyme S, 10 µl; crude toxin A, 10 µl, purified toxin A, 0.2 µg) were heated at 100° C for the indicated times. After diluting 10 fold in ice-cold 10 mM Tris-HCl (pH 7.4) the samples were assayed for ADP-ribosyl transferase activity as described in Materials and Methods, except that 5 µM [adenine-^{14}C]NAD^{+} (266 mCi/mmole) was used. Incubation was for 5 min.

Table 4. Comparison of the ADP-ribosyl transferase activity of exoenzyme S, Pseudomonas toxin A and diphtheria toxin fragment A with wheat germ extracts

Enzyme	ADP-Rib incorporated pmoles
Diphtheria toxin fragment A (0.1 µg)	29
Diphtheria toxin fragment A (0.2 µg)	33
Pseudomonas toxin A (0.1 µg)	33
Pseudomonas toxin A (0.2 µg)	32
Exoenzyme S (10 µl)	3670
None (10 µl H_2O)	0

Activity was determined as described in Materials and Methods with incubation for 15 min.

Table 5. Incorporation of [adenine-^{14}C]ADP-Rib into acid insoluble material with exoenzyme S and various substrates

Substrate	ADP-Rib Incorporated (pmoles)	
	Exoenzyme S	Toxin A
Wheat germ extract (160 µg)	3890	33
Reticulocyte extract (150 µg)	358	31
Reticulocyte EF-2 (76 µg)	0	370
Poly L-Lysine (50 µg)	0	0
BSA (50 µg)	0	0
Lysozyme (50 µg)	0	0
RNase (85 µg)	0	0
Rat Liver EF-1 (13 µg)	220	0
Reticulocyte EF-1 (20 µg)	45	0
H_2O (25 µl)	0	0

The reaction was carried out as described in Materials and Methods with 10 µl exoenzyme S and an incubation time of 15 min, with the following exceptions; both preparations of EF-1 were incubated for 60 min and the NAD^+ concentration was 0.14 mM (16.5 mCi/mmole).

Figure 1. Neutralization of the enzymatic activity of exoenzyme S and Pseudomonas toxin A by toxin A antiserum. (Δ) exoenzyme S; (●) Pseudomonas toxin A. Inactivation is expressed as the percentage of activity obtained when the toxin or enzyme was preincubated with normal rabbit serum (control).

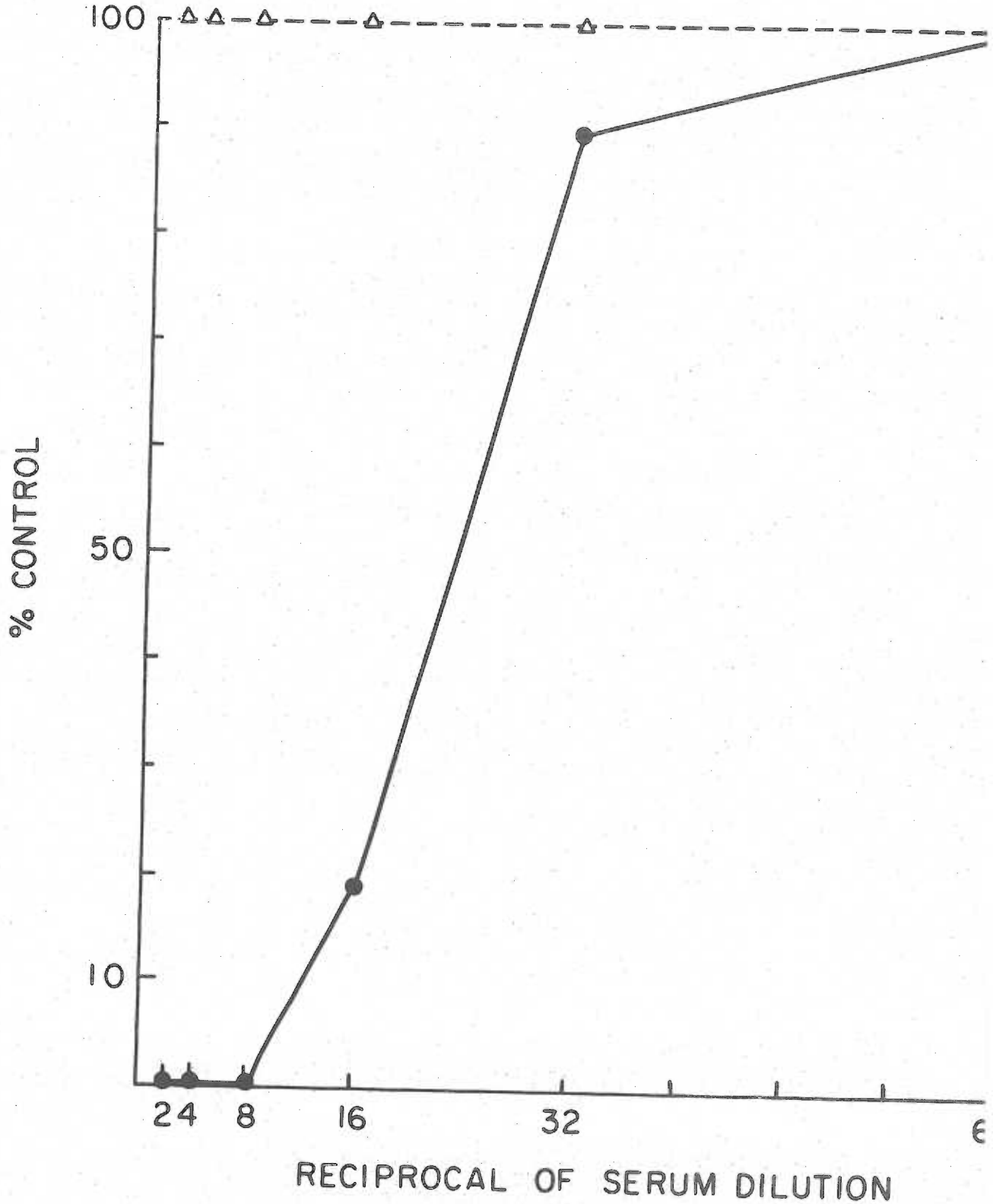
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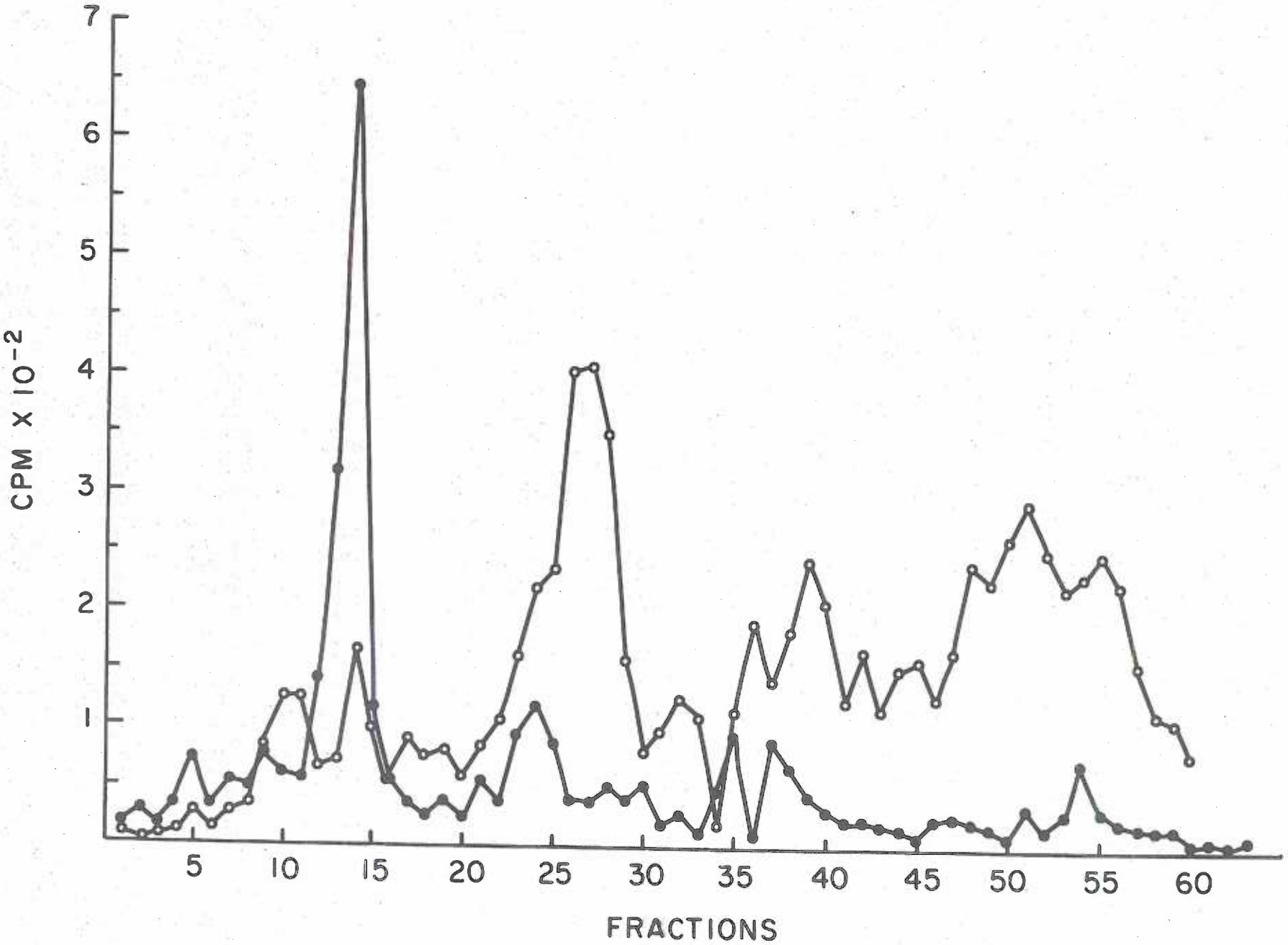
Figure 2. SDS-Polyacrylamide gel electrophoresis of acid-precipitable radiolabeled products formed in the presence of wheat germ extracts, 0.5 µg of Pseudomonas toxin A (●) or 1.0 µg exoenzyme S (○) and 5 µM [adenine-^{14}C]NAD^{+} (266 mCi/mmole).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Paper 5.

Production of exoenzyme S during Pseudomonas aeruginosa infections of burned mice.

Abstract

Antisera which could distinguish between Pseudomonas aeruginosa exoenzyme S and toxin could neutralize the adenosine diphosphate ribosyl (ADP-ribosyl) transferase activity of the homologous, but not the heterologous enzyme, and were used to identify the enzymatic activity in an unknown sample. Skin extracts and sera from burned mice infected with the exoenzyme S producing strain P. aeruginosa 388 contained ADP-ribosyl transferase activity that was not found in skin extracts or sera from uninfected mice. This ADP-ribosyl transferase activity was neutralized by S antiserum but not A antitoxin. The treatment of skin extracts or sera from burned infected mice with urea and dithiothreitol destroyed this enzymatic activity, which is consistent with the enzymatic properties of exoenzyme S but not toxin A. Thus, on the basis of immunological reactivity and enzymatic properties, the ADP-ribosyl transferase activity present in skin extracts and sera from P. aeruginosa 388 infected mice was identified as exoenzyme S. In addition, active EF-2 levels in burned mice infected with strain 388 were normal in the livers, kidneys and spleens at 24 h post-infection when compared to EF-2 levels in non-infected animals, indicating that strain 388 does not produce detectable amounts of toxin A in vivo. An unexpected finding in this report was the presence of S neutralizing activity in the sera from some non-immunized animals. Not only is exoenzyme S produced in vivo

during an experimental animal infection but pre-existing antibody suggests some animals had a previous exposure to this antigen.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that produces a wide variety of extracellular products that may contribute to its pathogenicity (17, 18). Toxin A, the most toxic substance known to be produced by P. aeruginosa (18), has the potential to be a major virulence factor (1, 3, 10, 11, 17, 19-25). Toxin A is produced in vitro by most clinical isolates of P. aeruginosa (3, 23), appears to be produced in humans during P. aeruginosa infections (22) and has been detected in skin extracts and sera from burned mice infected with a toxin A producing strain of P. aeruginosa (24). Specific A antitoxin has been shown to protect burned mice infected with some strains of P. aeruginosa (21, 25). Toxin A exerts its lethal effect by inhibiting protein synthesis in the same manner as diphtheria toxin; by catalyzing the transfer of the adenosine diphosphate ribose (ADPR) moiety of nicotinamide adenine dinucleotide (NAD) onto eukaryotic elongation factor 2 (EF-2), (6, 9, 10, 11).

A second extracellular protein (exoenzyme S) produced by some strains of P. aeruginosa has recently been shown to have ADP-ribosyl transferase activity (13). Exoenzyme S differs from toxin A in that S does not ADP-ribosylate EF-2 but rather modifies one or more different proteins present in eukaryotic cell extracts (13). Furthermore, exoenzyme S is not precipitated or neutralized by A antitoxin

(13). The enzymatic activity of S is partially destroyed by pretreatment with urea and dithiothreitol (DTT) (13) whereas such pretreatment potentiates the enzymatic activity of toxin A (16, 28).

While its enzymatic activity has at least partially been elucidated (13), no studies have been done to determine if exoenzyme S plays a role in P. aeruginosa infections. As a first step in evaluating this possibility, the present study was undertaken to determine if exoenzyme S is produced in vivo. A second objective was to further examine the immunological relationship between exoenzyme S and toxin A.

Materials and Methods

Bacterial strains. P. aeruginosa strain 388 was kindly provided by B. Minshew, University of Washington School of Medicine, Seattle, Wa. and strain PA-103 by P.V. Liu, University of Louisville School of Medicine, Louisville, Ky. Strain 388 has been shown to produce exoenzyme S, but not toxin A, in vitro (13). Strain PA-103 produces toxin A, but not exoenzyme S, in vitro. The strains were serotyped as described by Fisher et al. (7). Relevant characteristics of these strains are shown in Table 1.

Reagents. NAD ([^{14}C] adenine) was purchased from Amersham/Searle Corp. DTT, histamine, casein, elastin-congo red and nitrilotriacetic acid were purchased from Sigma Chemical Co. Norit A neutral-activated charcoal was obtained from Fisher Scientific.

Growth and exoenzyme S production by P. aeruginosa 388. The medium used for the growth of strain 388 was as previously described (13). An overnight culture (20 ml) was inoculated into 1 l of medium distributed evenly into ten 2 l Erlenmeyer flasks. The cultures were maintained at 32° C with vigorous shaking to increase aeration. Cell growth was monitored by reading the optical density at 540 nm in a Beckman spectrophotometer 20. At 22 h, the cells were removed by centrifugation at 10,000 x g for 20 min at 4° C. The culture supernatants were pooled.

Partial purification of exoenzyme S. Culture supernatant at 4° C was diluted with 3 volumes ice cold water and 50 g equilibrated diethylaminoethyl (DE-52) cellulose (Whatman, Inc., Clifton, N.J.) immediately added. The culture supernatant was stirred for 1 h and the DE-52 removed by filter suction onto Whatman No. 1 filter paper. The DE-52 was washed with 2 l of 50 mM NaCl, 10 mM tris(hydroxylmethyl)aminomethane-hydrochloride (Tris·HCl), pH 8.0, then exoenzyme S was eluted with 300 mM NaCl, 10 mM Tris·HCl, pH 8.0. The eluate was filter sterilized (Nalge Co., Rochester, N.Y.) and exoenzyme S concentrated by ultrafiltration using a PM-10 membrane (Amicon Corp., Lexington, Mass.). The buffer was reequilibrated to 50 mM NaCl, 10 mM Tris·HCl, pH 8.0 during the ultrafiltration. Approximately 15 ml (1.1 mg/ml) of this material was applied to a DEAE sephadex A-25 column (2.5 cm x 8.0 cm) previously equilibrated in the same buffer. A linear gradient from 50 mM NaCl to 400 mM NaCl was applied in 10 mM Tris·HCl, pH 8.0 and 5 ml fractions were collected. The major active

peak at 200 mM NaCl was pooled and concentrated on an Amicon PM-10 membrane. This partially purified exoenzyme S contained 0.48 mg protein/ml. The OD 280/260 ratio was 1.4. Aliquots were frozen at -70° C.

Production and purification of toxin A. P. aeruginosa PA-103 was used as a source of toxin A, which was produced and purified as previously described (28). The purified toxin A had a mouse median lethal dose (LD_{50}) of 0.2 µg/22 g mouse when injected intraperitoneally.

Preparation of specific antisera. The partially purified exoenzyme was used to immunize adult male New Zealand rabbits. A 1 ml mixture consisting of equal parts of Freunds complete adjuvant (FCA) and 200 µg/ml exoenzyme S in phosphate buffered saline (PBS) was injected into each rabbit as follows: 0.1 ml subcutaneously in each hindfoot, 0.4 ml subcutaneously in the back, and 0.4 ml intramuscularly. The animals were then injected three times at two week intervals using the same dose and sites, except Freunds incomplete adjuvant was substituted for FCA. Ten days after the last injection, the rabbits were bled by cardiac puncture and the separated serum (rabbit S antiserum) stored in small aliquots at -20° C. Purified toxin A was used to immunize rabbits and a sheep as previously described (12).

Preparation of toxin A coupled Sepharose 4B. One g cyanogen bromide activated Sepharose 4B (Pharmacia, Inc., Piscataway, N.J.) was suspended in coupling buffer (0.1 M $NaHCO_3$ containing 0.5 M NaCl). After 15 min at room temperature excess coupling buffer was removed

by filtration and the gel was suspended in 4 ml of pure toxin A (2 mg/ml) which had been previously dialysed against coupling buffer. Toxin A and activated Sepharose 4B were gently shaken at 4° C for 24 h in a plastic tube. Unbound toxin A was washed away with coupling buffer and the toxin A-coupled Sepharose (A-sepharose) was resuspended in 10 ml 1 M ethanolamine, pH 8.0 and shaken at room temperature for 2 h. The A-sepharose was then washed sequentially with 50 ml of 0.5 mM NaCl containing either 0.1 M sodium acetate, pH 4.0; 2 M urea; or 0.1 M sodium bicarbonate, pH 10.0. The A-sepharose was then resuspended in a buffer consisting of 50 mM Tris·HCl, pH 8.0, 200 mM NaCl, 1 mM ethylenediaminetetraacetate (EDTA) (column buffer) and packed into a 5 ml disposable syringe (2 ml bed volume) containing a porous plastic plug. When not in use, the column was equilibrated with column buffer containing 0.02% sodium azide.

Purification of toxin A antibody. The gamma globulin (IgG) was precipitated from the sheep antitoxin A with ammonium sulfate as previously described (12) then dialysed against column buffer and frozen as aliquots prior to column purification. Ten ml of this gamma globulin preparation were applied to the A-sepharose column for each run. All operations were performed at 4° C. Material unbound after 20 min at 4° C was washed out with column buffer, then bound material was eluted with 50 mM glycine·HCl, pH 3.2. The eluates (antitoxin A gamma globulin) from 3-4 columns were pooled and concentrated to 1 mg/ml on an Amicon PM-30 membrane, then reequilibrated with column buffer by ultrafiltration and stored at 4° C.

Enzyme neutralization by sera. All sera were heat inactivated (56° C for 15 min) prior to testing their ability to neutralize the enzymatic activity of exoenzyme S or toxin A. Crude culture supernatants of P. aeruginosa strains 388 and PA-103 were used as a source of crude S and A, respectively. Rate limiting concentrations of the enzymes were obtained by diluting crude S 1:30 and crude A 1:3 prior to their use. Crude A was then activated with urea and DTT as previously described (2). Neutralization was performed by incubating 5 µl of the appropriate serum with 5 µl of crude S or activated crude A for 15 min at 37° C. The neutralization mixture described above was incubated with 25 µl of wheat germ extract (150 µg), 25 µl of reaction buffer (5 mM Tris·HCl, pH 8.2, 0.1 mM EDTA, 40 mM DTT) and 5 µl NAD ([^{14}C] adenine) (280 mCi/mmol; 12.5 µCi/ml) for 5 min at 25° C. All reactions were stopped by the addition of 0.1 µl of 10% trichloroacetic acid (TCA) and the precipitates were collected, washed and radioactivity was measured as previously described (12).

Experimental burn infection model. A burned mouse model previously described (21, 26) was used. The model consists of the following process. Female Swiss white mice (strain NIH-NMRI CV) weighing 20±2 g were anesthesized with methoxyflurane (Penthrane; Abbott Laboratories, North Chicago, Ill.) and subjected to a 10 sec alcohol flame burn involving 15% of the total body surface. The challenge inocula were prepared as previously described (21). Mice were injected subcutaneously in the burn area immediately following burn trauma with 2 LD_{50} of the appropriate strain (approximately 3.6 x 10^4 PA-103;

2×10^2 for 388). This dose resulted in fatal infections in about 90% of the mice 50± 10 h post-infection. Control animals were anesthetized and injected subcutaneously with 0.5 ml sterile PBS or where indicated, mice were anesthetized, burned and then injected subcutaneously with sterile PBS. The burn trauma itself was not lethal.

At appropriate intervals post-infection, mice were sacrificed by cervical dislocation and blood obtained by cardiac puncture. Full thickness specimens of burned skin (or unburned skin from appropriate control animals) were removed and skin extracts prepared as described by Saelinger et al. (24).

Detection of ADP-ribosyl transferase activity in mouse skin extracts and sera. ADP-ribosyl transferase activity in skin extracts or sera from burned infected mice and control mice was measured using crude wheat germ extracts and NAD([^{14}C] adenine). Ten µl of skin extract or serum was incubated with 25 µl of wheat germ extract, 25 µl of reaction buffer and 5 µl of NAD([^{14}C] adenine) (280 mCi/mmol; 12.5 µCi/ml). The mixture was incubated for 30 min at 25° C. Neutralization of the ADP-ribosyl transferase activity in skin extracts and sera by A antitoxin and S antiserum was examined by preincubating 10 µl of sample with 10 µl of the appropriate serum for 15 min at 37° C and then measuring the ADP-ribosyl transferase activity. ADP-ribosyl transferase activity of urea and DTT treated skin extracts and sera was determined following preincubation of 10 µl of sample with 10 µl of a solution of 8 M urea and 2% DTT for 15 min at 25° C.

Extraction and quantitation of mouse organ EF-2. Livers, kidneys and spleens were removed from burned infected and control mice immediately after they were sacrificed and the tissues frozen at -70° C. EF-2 was extracted from homogenates of the organs by the method of Gill and Dinius (8) as modified by Iglewski et al. (11). The active EF-2 concentrations in the tissue extracts were determined using excess diphtheria toxin fragment A and NAD([^{14}C] adenine) as previously described (11).

Protein determinations. Protein concentrations were determined by the method of Bradford (4) modified by using a commercial reagent (Bio-rad Protein Assay Dye Reagent Concentrate) purchased from Bio-rad Lab., Richmond, Ca. Bovine gamma globulin (Bio-rad) was used as the standard.

Protease assays. Proteolytic activity in crude supernatants of P. aeruginosa strains PA-103 and 388 was determined by the method of Kunitz (14) as modified by Wretlind and Wadstrom (29) using casein as the substrate.

Elastase activity was quantified using elastin-congo red as the substrate. The reaction mixture consisted of Tris-maleate buffer (0.1 M, pH 7.0) supplemented with $CaCl_2$ (1 mM) (buffer A). One ml of culture supernatant was added to 2 ml of buffer A containing 10 mg elastin-congo red. The reaction was carried out in stoppered 15 ml centrifuge tubes incubated 2 h in a 37° C water bath with rapid shaking. The reaction was terminated by the addition of 2 ml sodium phosphate buffer (0.7 M, pH 6.0). The precipitate was removed by centrifugation. The blank consisted of 3 ml buffer A containing 10 mg

elastin-congo red. Elastase activity was determined by reading absorbance of the supernatants at 495 nm in a Beckman spectrophotometer 20.

Results

Specific neutralization of exoenzyme S activity. To determine if exoenzyme S was produced in vivo, it was first necessary to develop methods to specifically identify this enzyme and distinguish it from toxin A. An immunological approach was taken. Partially purified exoenzyme S was used to immunize rabbits. Antisera obtained from two separate rabbits neutralized the enzymatic activity of exoenzyme S but not toxin A (Table 2). While both rabbits produced S antibodies following immunization with exoenzyme S, they did not respond equally. The enzymatic activity of exoenzyme S was not neutralized by A antitoxin raised in rabbits immunized with pure toxin A. This A antitoxin completely neutralized the toxin A enzymatic activity (Table 2 and ref. 13). Thus these antisera specifically neutralized the enzymatic activity of the homologous, but not the heterologous, enzyme and they could be utilized to identify the enzymatic activity in an unknown sample.

We also tested the neutralizing ability of A antitoxin which had been raised in sheep by immunization with pure toxin A. Surprisingly, this sheep antitoxin A (sheep #1, Table 3B) neutralized the enzymatic activity of both A and S. However, when examined, it was found that the prebleed serum from this sheep also neutralized S enzymatic

activity, but not toxin A enzymatic activity (Table 3A). Anti-S activity purified along with gamma globulin but did not co-purify with specific antitoxin A gamma globulin when it was purified on a toxin A-sepharose 4B affinity column (Table 3B). Anti-S was also found in other (4 of 6) normal sheep sera, 1 of 5 normal rabbit sera and 2 of 6 normal mouse sera (Table 3A). It is interesting that none of the normal sera tested neutralized the enzymatic activity of toxin A (Table 3A). These results indicated that many non-immunized animals had preexisting antibodies that neutralized exoenzyme S activity.

Infection of burned mice with strain 388. The LD_{50} of strain 388 was markedly reduced when mice were previously subjected to the experimental thermal injury (Table 1). The LD_{50} of strain 388 in normal (unburned) mice was 2.0×10^6 organisms, in contrast to a LD_{50} of 1.1×10^2 organisms in a burned mouse. This represents an approximate 20,000 fold reduction in the LD_{50}. In the remaining experiments, we used a challenge inoculum of 2 LD_{50}'s in the burned mice.

In vivo production of exoenzyme S. Skin extracts from burned mice that were infected with P. aeruginosa strain 388 contained ADP-ribosyl transferase activity that was not found in skin extracts from uninfected control mice (Fig. 1). The enzyme activity was present in the burned infected mouse skin extracts at the earliest time post-infection (18 h) that we tested. The mean ADP-ribosyl transferase activity in the burned infected skin extracts remained

relatively constant from 18 h to 48 h post-infection. There was a wide range of ADP-ribosyl transferase levels found in the skin extracts of individual burned infected mice. However, skin extracts from 37 of 39 infected mice had enzyme levels higher than those of skin extracts from all 49 control animals (Fig. 1) ($p < 0.01$).

Sera from burned infected mice contained ADP-ribosyl transferase activity (Fig. 2). Sera from control non-infected mice also contained endogenous ADP-ribosyl transferase activity consistent with a previous report (24). At 18 h post-infection the average level of ADP-ribosyl transferase activity in the sera from burned infected mice was equal to that in the sera from control non-infected mice (Fig. 2). Levels of ADP-ribosyl transferase activity in the sera from infected mice increased markedly at 24 h post-infection and continued to increase linearly through 48 h. The average enzyme levels in sera from non-infected control mice did not change significantly over the 48 h period (Fig. 2).

Identification of the ADP-ribosyl transferase activity in samples from burned infected mice. The ADP-ribosyl transferase activity in skin extracts and sera from burned infected mice was further characterized. This enzyme activity was partially neutralized by S antiserum but not A antitoxin (Table 4A and B). The ADP-ribosyl transferase activity in the skin extracts and sera of infected mice that was not neutralized by anti-S serum perhaps represents an endogenous enzyme. The ADP-ribosyl transferase activity in sera from control (non-infected) mice was not neutralized by either A

antitoxin or S antiserum (Table 4C and D). These data indicate that most of the ADP-ribosyl transferase present in skin extracts and sera of infected mice was due to exoenzyme S and that present in sera of control mice was due to an endogenous enzyme.

The treatment of skin extracts or sera from burned infected mice with urea and DTT partially destroyed the enzymatic activity (Table 5B and C). To determine if the skin extracts or sera contained a factor which might alter these enzymes, crude exoenzyme S or crude toxin A was pre-incubated in skin extracts or sera from burned non-infected mice at 25° C for 15 min prior to assaying their enzymatic activities. The pre-incubation of these enzymes in normal mouse sera or skin extracts did not alter their enzymatic properties (Table 5A).

Active EF-2 levels in organs from P. aeruginosa 388 infected burned mice. Infection of burned mice with toxin A producing strains of P. aeruginosa has been shown to cause early and rapid decreases in the active EF-2 content of various tissues, especially the liver (20, 21, 25). In contrast, the active EF-2 content of tissues from burned mice infected with a non-toxinogenic (toxin A^-, S^-) strain of P. aeruginosa (WR-5) remained normal, then decreased slightly just prior to death (20). In agreement with these previous reports, the levels of active EF-2 in tissues from burned mice infected with strain PA-103 were markedly decreased at 24 h post-infection (Table 6). The greatest effect was seen in the liver where active EF-2 levels were decreased to 35% of the control levels. In contrast, active EF-2 levels in burned mice infected with strain 388 were normal in the livers, kidneys and spleens at 24 h post-infection when compared to

EF-2 levels in tissues from control mice (Table 6). At 48 h post-infection, only small decreases in the active EF-2 levels in the livers were found in 388 infected animals moribund and near death (data not shown). The small decreases in active EF-2 levels observed at 48 h post-infection were similar to those previously reported for the toxin A and S negative strain WR-5 (20).

Discussion

Exoenzyme S is a recently discovered ADP-ribosyl transferase, produced by some strains of P. aeruginosa, that is distinctly different from toxin A (13). Exoenzyme S catalyzes the ADP-ribosylation of one or more proteins present in eukaryotic cell free extracts but unlike toxin A, S does not ADP-ribosylate EF-2 (13). The enzymatic activity of exoenzyme S, in contrast to toxin A, is destroyed rather than potentiated by pretreatment with urea and DTT and the enzymatic activity of S is not neutralized by A antitoxin (13).

An unexpected finding in this investigation was the presence of S neutralizing activity in the sera of some non-immunized animals. The sera from one of five rabbits, 5 of 7 sheep and 2 of 6 mice partially neutralized S enzymatic activity (Table 3A). One of the sheep that was immunized with purified toxin A showed anti-S activity in the prebleed and in the immune (antitoxin A) serum. The anti-S neutralizing activity in this serum co-purified with gamma globulin but did not co-purify with specific antitoxin A gamma globulin

(Table 3B). These results indicate that the anti-S neutralizing activity in the normal sera was due to immunoglobulin molecules that recognized S but not A. In addition, the lack of binding of the anti-S activity to the toxin A sepharose 4B affinity column (Table 3B), confirms the lack of cross-neutralization between A antitoxin and S antiserum for the heterologous enzyme (Table 2). It is possible that the anti-S activity in the pre-immune sera of these animals was due to the production of specific antibodies to exoenzyme S following a previous encounter with S producing strains of P. aeruginosa, or to antibodies directed at antigens which cross-react with S.

Our finding of S neutralizing activity in pre-immune sera from a variety of animals is significant since several studies utilized antitoxin raised to A to identify toxin A producing strains of P. aeruginosa (3, 23), for purifying toxin A (12, 27) and to monitor toxin A purification (5, 12, 15). In light of our results (Table 3), A antitoxin or prebleed serum should not be used to detect A until the anti-S effects have been accounted for. Otherwise, unwarranted interpretations of the data may occur when using the antisera to test for the presence of toxin A.

By immunizing only rabbits whose prebleed sera contained no detectable S or A antibodies we were able to develop a suitable S antiserum. In a previous report (13), the enzymatic activity of exoenzyme S was not neutralized by A antitoxin. This observation is confirmed in this report and it is also demonstrated that the enzymatic activity (Table 3B) of toxin A is not neutralized by exoenzyme S antibody (Table

2). Thus, in these enzyme neutralization assays there is no cross-reaction between antitoxin A and S antiserum and these specific antisera can be used to identify the enzymatic activity in an unknown sample.

Most extracellular bacterial products known to be virulence factors have been shown to be produced *in vivo*. We attempted to detect the *in vivo* production of exoenzyme S by *P. aeruginosa* strain 388, a strain that produces S *in vitro* (13). We utilized the burned mouse model of Stieritz and Holder (26) as modified by Pavlovskis et al. (21) to demonstrate the *in vivo* production of *P. aeruginosa* toxin A. The model was suitable for this study because the bacterial strain (*P. aeruginosa* 388) used in the present study was originally isolated from a burned wound (Table 1) and at least one of the aspects of this model, toxemia, resembles clinical *Pseudomonas* burn sepsis (26). In addition, the model requires realistically small infecting doses, reflecting an actual clinical situation (26).

Exoenzyme S was produced *in vivo* in burned mice infected with *P. aeruginosa* 388 (Fig. 1 and 2). ADP-ribosyl transferase activity was detected in extracts of skin obtained 18, 24, 36 and 48 h post-infection (Fig. 1). This enzymatic activity was also detected in sera from burned mice infected with strain 388 at 24 h post-infection and the mean levels increased approximately linearly through 48 h (Fig. 2). That the ADP-riboysl transferase activity detected in the skin extracts and sera of burned infected mice was indeed due to exoenzyme S was shown by its specific neutralization by S antiserum

but not A antitoxin (Table 4). In addition, this enzymatic activity present in skin extracts and sera from 388 infected animals was decreased by pretreatment with urea and DTT (Table 5) which is characteristic of exoenzyme S but not toxin A (13). Finally, in contrast to the reduction of active EF-2 levels in tissues from burned mice infected with toxin A producing strains of P. aeruginosa (20, 24) levels of EF-2 in the livers, kidneys and spleens of burned mice 24 h after infection with strain 388 were not altered in comparison to the levels of EF-2 in non-infected control mice (Table 6). These data (Table 4, 5 and 6) indicate that strain 388 does not produce detectable amounts of toxin A in vivo. Small decreases in active EF-2 levels were observed in tissues from animals infected 48 h previously with strain 388. These slight decreases seen with strain 388 were similar to decreases previously reported using the toxin A and S negative strain, WR-5, and perhaps reflect non-specific tissue degeneration in moribund animals (20).

Exoenzyme S levels in skin extracts and sera of the burned infected mice varied over a wide range (Fig. 1 and 2). When the sera of 6 normal mice were tested for S neutralizing activity, 2/6 were capable of partially neutralizing the ADP-ribosyl transferase activity of exoenzyme S (Table 3). Thus, one explanation for the wide range of responses of individual animals could be the presence of pre-existing antibodies.

In conclusion, we have shown that exoenzyme S, but not toxin A, is produced in vivo in animals infected with P. aeruginosa strain 388. Furthermore, strain 388 was virulent for burned mice.

Thus, exoenzyme S may be a virulence factor of P. aeruginosa. However, information concerning its toxicity, its production by clinical isolates and the protective capabilities of specific S antibodies in P. aeruginosa infections is required to evaluate the relative importance of exoenzyme S.

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Table 1. Characterization of P. aeruginosa strains 388 and PA-103

Strain	Source	Serotype	Toxin A	Exoenzyme S	Protease	Elastase	LD_{50}(CFU)[a] Normal Mice	LD_{50}(CFU)[a] Burned Mice
388	burn wound	1	-	+	+	+	2.0×10^6	1.1×10^2
PA-103	sputum	2	+	-	+	-	1.8×10^6	1.2×10^3

[a]CFU, colony forming units

Table 2. Neutralization of exoenzyme S and toxin A by rabbit antisera[a]

	% Neutralization[b]			
Enzyme	S antiserum #1	S antiserum #2	A antitoxin #1	A antitoxin #2
Exoenzyme S	95	55	0	0
Toxin A	0	0	100	100

[a]Prebleed sera from the rabbits in which these antisera were raised contained no detectable S or A antibodies.

[b]Neutralization is expressed as the percentage of ADP-ribosyl transferase activity obtained when exoenzyme S or toxin A was incubated with an equal volume of 0.9% saline containing 0.1 mg/ml BSA for 15 min at 37° C.

Table 3. Neutralization of toxin A and exoenzyme S enzymatic activity with normal animal sera and sheep antitoxin A

A. Normal sera		Neutralization[a]	
Animal species	Serum no.	Exoenzyme S	Toxin A
Rabbit	1	-	-
	2	-	-
	3	+ (32)	-
	4	-	-
	5	-	-
Mouse	1	+ (20)	-
	2	+ (21)	-
	3	-	-
	4	-	-
	5	-	-
	6	-	-
Sheep	1	+ (91)	-
	2	+ (84)	-
	3	-	-
	4	-	-
	5	+ (53)	-
	6	+ (29)	-
	7	+ (33)	-
B. Sheep antitoxin A[b]			
Whole serum		+ (91)	+(100)
Gamma globulin		+ (89)	+(100)
Antitoxin A gamma globulin		-	+(100)

[a]Numbers in parenthesis represent percent of exoenzyme S or toxin A ADP-ribosyl transferase activity that was neutralized by the serum, as compared to the ADP-ribosyl transferase activity of a sample treated with an equal volume of 0.9% saline containing 0.1 mg/ml BSA for 15 min at 37° C.

[b]This antitoxin A was raised in sheep #1.

Table 4. Neutralization of ADP-ribosyl transferase activity in skin extracts and sera from mice

	Neutralization[a]	
	A antitoxin	S antiserum
A. Skin extracts: 388[b] infected burned mice		
Skin no.		
3	-	+(87)
22	-	+(84)
23	-	+(82)
39	-	+(86)
56	-	+(87)
B. Sera: 388 infected burned mice		
Serum no.		
21	-	+(53)
37	-	+(80)
38	-	+(78)
40	-	+(57)
41	-	+(73)
C. Sera: control (burned non-infected mice)		
Serum no.		
45	-	-
47	-	-
D. Sera: control (anesthesized only) mice		
Serum no.		
31	-	-
50	-	-

[a]Numbers in parenthesis represent percent of skin extract or serum ADP-ribosyl transferase activity that was neutralized by A antitoxin or S antiserum as compared to the ADP-ribosyl transferase activity of a sample treated with an equal volume of 0.9% saline containing 0.1 mg/ml BSA for 15 min at 37° C.

[b]Strain 388 produced exoenzyme S but undetectable levels of toxin A.

Table 5. Effect of urea and dithiothreitol on the ADP-ribosyl transferase activity of mouse skin extracts and sera

	ADPR incorporated (pmole)[a]	
	$+H_2O$	+urea, DTT
A. Control skin extracts or sera + toxin A or exoenzyme S[b]		
Skin extract + toxin A	0.8	12.4
Skin extract + exoenzyme S	8.5	4.9
Serum + toxin A	1.7	13.4
Serum + exoenzyme S	11.9	5.5
B. Skin extracts from burned infected mice		
Skin no.		
3	28.0	15.4
23	48.1	32.1
37	60.0	45.9
39	50.9	29.0
56	25.9	19.6
C. Sera of burned infected mice		
Serum no.		
19	14.3	6.8
21	13.3	8.2
23	9.5	6.5
37	26.7	17.0
41	12.1	4.5

[a]Per 10 µl of skin extract or serum.

[b]Crude toxin A (10 µl) or crude exoenzyme S (10 µl of a 1:10 dilution) were pre-incubated with 90 µl of skin extract or sera for 1 h at 37° C before being tested for ADP-ribosyl transferase activity.

Table 6. Comparison of the active EF-2 levels in tissue extracts from burned mice infected with *P. aeruginosa* PA-103 or 388[a]

	% Control active EF-2 levels[b]	
Organ[c]	PA-103 infection[d]	388 infection[e]
Liver	35	102
Kidney	83	95
Spleen	82	101

[a]Mice were sacrificed 24 h after being burned and infected.

[b]Control values were obtained using the appropriate tissue from anesthetized and burned uninfected mice.

[c]Organs from 6 similarly treated mice were pooled.

[d]Strain PA-103 produced toxin A but undetectable levels of exoenzyme S.

[e]Strain 388 produced exoenzyme S but undetectable levels of toxin A.

Figure 1. ADP-ribosyl transferase activity in skin extracts of burned mice infected with P. aeruginosa strain 388 and skin extracts of control non-infected mice that were anesthetized and burned or anesthetized only. The horizontal lines represent the mean ADP-ribosyl transferase activity or skin extracts for each group of mice.

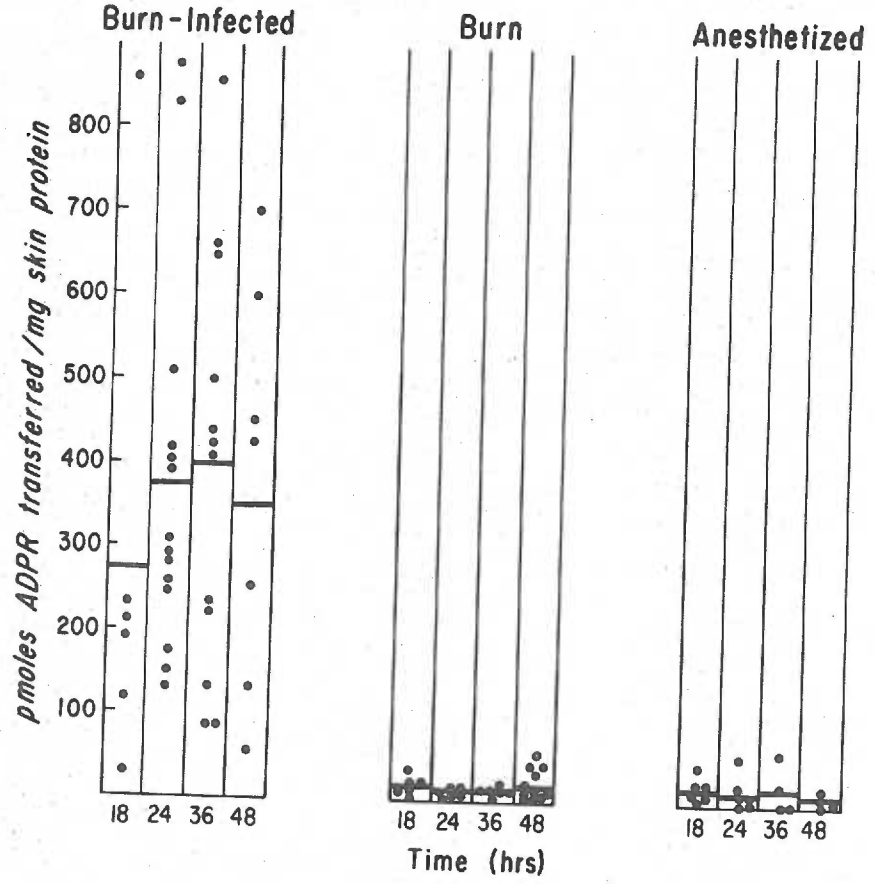
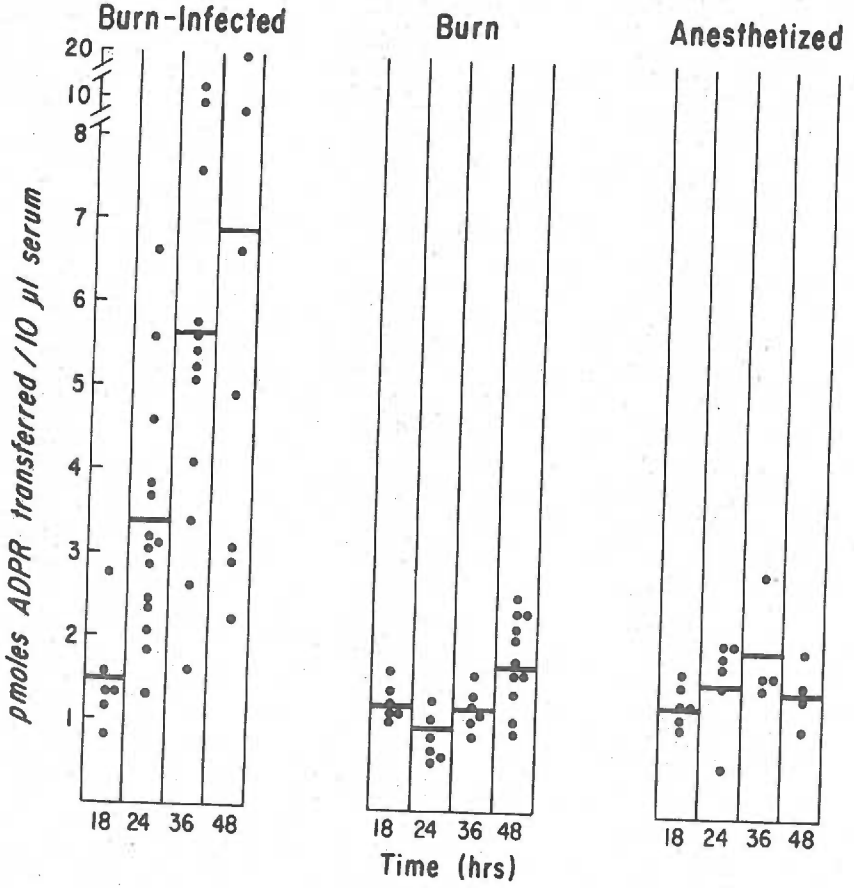

Burn-Infected
Burn
Anesthetized
pmoles ADPR transferred/mg skin protein
800
700
600
500
400
300
200
100
18 24 36 48
18 24 36 48
18 24 36 48
Time (hrs)

Figure 2. ADP-ribosyl transferase activity in sera from burned mice infected with P. aeruginosa strain 388 and in sera from control non-infected mice that were anesthetized and burned or anesthetized only. The horizontal lines represent the mean ADP-ribosyl transferase activity of sera from each group of mice.


Burn-Infected
Burn
Anesthetized
pmoles ADPR transferred / 10 µl serum
20
10
8
7
6
5
4
3
2
1
18 24 36 48
18 24 36 48
18 24 36 48
Time (hrs)

Paper 6.

Factors that influence the production of Pseudomonas aeruginosa exoenzyme S.

Abstract

A medium has been developed and culture conditions identified which result in high yields of exoenzyme S in liquid cultures of Pseudomonas aeruginosa strain 388. The optimum medium composition and culture conditions for S were found to be similar but not identical to those previously reported for toxin A production (15). The major difference between S and A production was that S production required the presence of a chelating agent, either nitrilotriacetic acid (NTA) or ethylenediamenetetraacetate (EDTA). The optimum concentration of NTA or EDTA was found to be 10 mM. Furthermore, bovine serum albumin (BSA), either partially purified or crystalline, markedly inhibited S yields whereas crystalline BSA has previously been shown to enhance toxin A yields (15). The addition of various amino acids resulted in increased S yields but monosodium glutamate (MSG) (100 mM) gave the highest S yields. Yields of S were increased by the addition of glycerol to 1%. As the iron concentration of the growth medium was increased to 2 µg/ml and above, S yields were markedly (80%) decreased. A low iron medium composed of a dialysate of trypticase soy broth supplemented with 10 mM NTA, 1% glycerol and 100 mM MSG gave the highest yields of S. Relatively high S yields were also obtained when strain 388 was cultured in some other basal medium similarly supplemented. The optimum culture conditions for high S yields were adequate aeration and a temperature of 32° C. Under these culture conditions exoenzyme S was first detectable at 10 h and maximum yields were found at 20-24 h.

Introduction

Exoenzyme S is a recently discovered adenosine diphosphate ribosyl (ADP-ribosyl) transferase that is produced by some strains of Pseudomonas aeruginosa (11). This enzyme catalyzes the transfer of the adenosine diphosphate ribose (ADPR) moiety of nicotinamide adenine dinucleotide (NAD^+) onto proteins present in extracts of eukaryotic cells (11). However, exoenzyme S does not ADP-ribosylate elongation factor 2 (EF-2) as does P. aeruginosa toxin A (8, 9, 11). The ADP-ribosyl transferase activity of exoenzyme S is not neutralized, nor the enzyme precipitated, by toxin A antibody (11). In addition, the enzymatic activity of exoenzyme S differs from toxin A in that S activity is destroyed rather than potentiated (as is toxin A's enzymatic activity) by pretreatment with urea and dithiothreitol (11).

Toxin A is not formed constitutively by A toxinogenic strains of P. aeruginosa. Aeration, a temperature of 32° C, glycerol and monosodium glutamate (MSG) are required to obtain optimal yields of toxin A (15). Nucleic acids and iron inhibit the production of toxin A but enhance the growth of P. aeruginosa (2, 15). While the factors that regulate yields of toxin A have been studied, little is known about the regulation of exoenzyme S yields. The aim of this study was to identify factors which regulate the yields of exoenzyme S in cultures of P. aeruginosa and optimize the culture conditions and medium constituents.

Materials and Methods

Microorganisms. P. aeruginosa strain 388 was used throughout this study. Strain 388, the kind gift of B. Minshew, University of Washington, Seattle, Wa., was stored frozen in 10% skim milk at -70° C.

Reagents. NAD([^{14}C] adenine) at 280 mCi/mmol was purchased from Amersham/Searle Corp. Nitrilotriacetic acid (NTA), bovine serum albumin ([BSA]; both fraction V and crystalline), casein, monosodium glutamate (MSG), elastin-congo red and tricine (N-Tris[hydroxymethyl]methylglycine) were purchased from Sigma Chemical Co., St. Louis, Mo. Glycerol and citrate were purchased from Fisher Scientific Co., Fair Lawn, N.J. and ethylenediaminetetraacetate (EDTA) from Matheson, Coleman and Bell, Los Angeles, Ca.

Media and culture conditions. Trypticase soy broth (TSB) medium was prepared by dissolving 30 g trypticase soy broth (BBL, Cockeysville, Md.) in 1 l of distilled water. Dialysed TSB (TSBD) was prepared as described by Liu (15). For one set of experiments, TSBD medium was deferrated and the residual iron concentration of the medium determined (20). Syncase medium was prepared as described by Finkelstein et al. (7) with the exception that no iron was added. A proteose peptone (PP) medium was prepared as described by Pope (21). To prepare dialysed proteose peptone (PPD) medium, 20 g of protease peptone powder (Difco Lab, Detroit, Mich.) dissolved in 60 ml H_2O was dialysed against 1 l H_2O. The other components of Pope's medium, $MgSO_4$, 0.2 mg; Na_2HPO_4, 1.0 g; K_2HPO_4, 1.0 g; glucose, 2.0 g; and sodium lactate (12 ml of a 50% solution) were then added

to each liter of dialysate. PGT (pantothenate, glutamic acid and tryptophan) medium was prepared as described by Barksdale and Pappenheimer (1). Unless otherwise indicated, media were supplemented with 10 mM NTA, adjusted to pH 7.0, autoclaved, then sterile solutions of MSG and glycerol were added to a final concentration of 100 mM and 1%, respectively.

Bacterial growth was measured by removing a portion of the culture and reading the optical density at 540 nm (OD_{540}) in a Beckman spectrophotometer 20. Generally, the culture conditions were as follows: 10 ml of the appropriate medium in a 125 ml Erlenmeyer flask was inoculated with a 15 h culture of strain 388 to give 0.05 OD_{540} units. Incubation was for 20 h at 32° C in a reciprocating shaking water bath (Lab-line Instruments, Melrose, Ind.).

ADP-ribosyl transferase assay. Aminoacyl transferase-containing enzymes were prepared from wheat germ as described by Chung and Collier (6). The ADP-ribosyl transferase assay (11) was performed at 25° C for 5 min in a mixture containing 25 µl of wheat germ extract, 25 µl of reaction buffer (5 mM Tris[hydroxymethyl]aminomethane-hydrochloride [Tris·HCl], pH 8.0), 5 µl of ([^{14}C] adenine) NAD at 280 mCi/mmol (12.5 µCi/ml) and 5 µl of a 1:10 dilution of crude culture supernatant in 0.9% saline. The reaction was stopped by the addition of 0.1 ml of 10% trichloroacetic acid. The precipitates were collected, washed and radioactivity was measured (10).

Protease assay. Protease activity in culture supernatants was determined by a previously described (22) modification of the method of Kunitz (13) using casein as the substrate.

Results

Effect of NTA on exoenzyme S yields and protease activity. Callahan has previously shown that the presence of NTA in the culture medium inhibits the enzymatic activity of P. aeruginosa proteases (5). For this reason, NTA was added to TSBD medium in an attempt to detect toxin A in cultures of Elek negative (3) strains of P. aeruginosa. Some of the strains, in the presence of NTA, produced an ADP-ribosyl transferase (exoenzyme S) that was distinct from toxin A (11).

The effect of varying the NTA concentration of the medium (TSBD) on bacterial growth and exoenzyme S yields was investigated (Fig. 1). NTA (0-20 mM) did not affect bacterial growth. At NTA concentrations greater than 40 mM there was a slight decrease in the final yields of bacteria. In contrast to its minimal effect on growth, NTA had a dramatic effect on S yields. When NTA was omitted from the culture medium, ADP-ribosyl transferase activity was barely detectable (Fig. 1). Addition of from 1 mM to 10 mM NTA to TSBD medium increased S yields, while NTA concentrations greater than 20 mM decreased S yields. However, S activity was still detectable at concentrations of NTA as high as 100 mM (Fig. 1). While there was a broad peak of NTA concentrations (5-20 mM) at which high yields of S were produced, the optimum concentration of NTA was 10 mM (Fig. 1).

In light of Callahan's previous report (5), it seemed possible that NTA increased S yields by inhbiting the enzymatic activities of P. aeruginosa proteases, which might otherwise degrade S. To test this possibility, the effect of varying concentrations of NTA in the medium

on S yields and protease activity was examined. As the concentration of NTA in the medium was increased, the protease activity decreased while S yields increased (Fig. 2). Addition of NTA to 4 mM resulted in a complete loss of detectable protease activity. However, S yields continued to increase as the concentration of NTA increased from 4 through 10 mM (Fig. 1 and 2).

Ammonium sulfate [$(NH_4)_2SO_4$] has also been used to inhibit the activity of proteases in cultures of P. aeruginosa (16). When ammonium sulfate (1-400 mM) was added to TSBD medium in place of NTA, only barely detectable yields of exoenzyme S were found (data not shown).

Since NTA is a potent chelating agent (5, 17) the effect of other chelating agents on S yields was examined (Table 1). The chelating agents tested were tricine, citrate and EDTA. The addition of (0.1 mM-20 mM) tricine or citrate to the medium in place of NTA resulted in negligible S yields. However, the addition of EDTA to the medium resulted in S yields equal to the yields obtained when 10 mM NTA was present (Table 1 and Fig. 1). However, EDTA inhibited bacterial growth and pigment production by strain 388. The concentration of NTA or EDTA which gave maximum S yields was 10 mM and in subsequent experiments the media were supplemented with 10 mM NTA.

Effect of amino acids on S yields. A number of amino acids were added to TSBD to determine their effect on S yields. Among those tested were alanine, arginine, aspartic acid, glycine, isoleucine, and glutamic acid. Of these amino acids, aspartic acid and glutamic

acid resulted in maximal S yields when added to TSBD medium, while the addition of arginine gave 50% maximal yields and alanine, glycine or isoleucine resulted in S yields that were 10-20% maximal (data not shown). These amino acids are expensive and some of them are difficult to dissolve. A substitute, MSG, was found that was both inexpensive and water soluble at high concentrations. Increasing the concentration of MSG in the medium from 0 mM to 50 mM resulted in a substantial increase in bacterial growth (Fig. 3). At concentrations above 50 mM, MSG had no obvious effect on growth. The addition of MSG to TSBD medium increased S yields. As the concentration of MSG in the medium increased from 10 to 100 mM, S yields increased proportionally (Fig. 3). At concentrations greater than 100 mM, MSG had an inhibitory effect on S yields although even at 200 mM the yields were still about 40% maximal (Fig. 3).

To determine if protein could substitute for MSG, the effect of various concentrations of BSA (fraction V or crystalline) on S yields and bacterial growth was determined. The addition of either partially pure (fraction V) or crystalline BSA in place of MSG resulted in decreased yields of exoenzyme S, while at the same time stimulating bacterial growth (Fig. 4). The data showing the inhibitory effects of fraction V and crystalline BSA were very similar (Fig. 4). In light of these results (Fig. 3 and 4), MSG at its optimum concentration of 100 mM was added to media used in subsequent experiments.

Effect of glycerol on S yields. Because glycerol had previously been found to enhance yields of toxin A (15), in previous experiments we added glycerol (1% final concentration) to the growth medium. To

determine if glycerol stimulated S yields and if so to determine its optimum concentration, the effect of various concentrations of glycerol on S yields and bacterial growth was examined. The addition of glycerol to a final concentration of 0.25% markedly increased bacterial growth but had no effect on S yields (Table 2). Glycerol at a final concentration of 0.5% doubled the S yields and at 1.0%, S yields were maximal. In the presence of 2.0% glycerol the S yields decreased (Table 2). Glycerol (1%) was added to media for all subsequent experiments.

Effect of iron on S yields. Since the yields of a number of bacterial products, including P. aeruginosa pigments and toxin A, are decreased by increasing the iron concentration of the medium (2, 4, 12), the effect of iron on S yields in cultures of strain 388 was examined. Increasing the concentration of iron in the medium resulted in an increase in bacterial growth (Fig. 5). In contrast, as the iron concentration of the medium increased from 0.05 µg/ml to 1.0 µg/ml the yields of exoenzyme S decreased. No further decreases in S yields occurred when the concentration of iron was increased above 2.0 µg/ml. Exoenzyme S yields in medium containing 2 or 5 µg iron/ml were decreased by about 80% as compared to medium containing 0.05 µg/ml (Fig. 5).

Culture conditions and S yields. Bacterial growth and S yields were both increased in shaking cultures of strain 388 as compared to stationary cultures (Table 3). Bacterial growth was increased 2-3 fold in the shaking cultures while exoenzyme S yields were increased about 50-fold as compared to yields in stationary cultures. The optimum temperature of incubation for production of S yields in stationary cultures

was 32-35° C. In shaking cultures the optimum was 32° C and 80% maximal S yields were found at 30° C. Yields of S in shaking cultures incubated at 25°-37° C were higher than S yields in stationary cultures even when the latter were incubated at their optimum temperatures (32-35° C) (Table 3).

Rate of exoenzyme S release and bacterial growth. Strain 388 was cultured in TSBD medium supplemented with 10 mM NTA, 100 mM MSG and 1% glycerol. At 2 h intervals samples were removed from the culture and examined for growth and exoenzyme S yields (Fig. 6). The generation time of strain 388 during exponential growth was 25 min as calculated by the method of Miller (18). Strain 388 reached stationary phase at 18 h. Exoenzyme S was first detected at 10 h and the rate of release remained approximately constant through 20 h. Maximal yields of S were found late in the bacterial growth cycle at 20-24 h (Fig. 6).

Other media and S yields. Yields of exoenzyme S in other media were compared to those in TSBD medium (Table 4). Some of these media, such as syncase, PGT and PP have been used for the production of other bacterial toxins (1, 7, 21). Of the media tested, S yields were maximal in TSBD. However, relatively high S yields were obtained when strain 388 was cultured in some of the other media. The dialysate of TSBD resulted in much higher yields of S than TSB medium. Conversely, PP medium (non-dialysed) resulted in higher S yields than PPD (Table 4). When NTA was omitted from any of the media, S activity was barely detectable (data not shown). Exoenzyme S yields were not

simply a function of bacterial growth since there was no consistent relationship between bacterial yields and S yields in the media tested (Table 4).

Discussion

A medium has been developed which results in reproducible high yields of exoenzyme S in cultures of P. aeruginosa strain 388. This medium (Table 5) is relatively inexpensive, easily prepared and has the advantage that it contains no high molecular weight components that could otherwise complicate the purification of exoenzyme S. Although the medium was optimized for S yields using strain 388, other exoenzyme S producing strains of P. aeruginosa produce high S yields in this medium (data not shown). Routinely, a flask to volume ratio of at least 10 to 1 was used to obtain adequate aeration. Furthermore, yields of exoenzyme S were approximately 50 times higher in shaking cultures than in stationary cultures (Table 3). Exoenzyme S yields consistent with the yields described in this paper have also been obtained in fermentor scale cultures of strain 388 using the fermentation conditions described by Leppla for toxin A production (14). The optimum incubation temprature was 32° C, although significant amounts of S were obtained in shaken cultures incubated between 30-37° C (Table 3).

The medium and conditions that have been developed for optimizing yields of exoenzyme S (Table 5) are similar to those developed by Liu for toxin A (15). Among the similarities are the use of TSBD as

the base medium, the requirement for MSG and glycerol, and culture conditions such as the temperature of incubation and aeration. Furthermore, maximum yields of S are obtained in growth medium containing relatively low concentrations of iron and yields decrease in the presence of high iron concentrations (Fig. 5). A similar relationship has previously been shown for iron and toxin A yields (2). In addition to having similar requirements for growth medium composition and culture conditions, the initial appearance and rate of release of exoenzyme S (Fig. 5) are similar to that previously shown for toxin A (2).

Differences were observed in the regulation of exoenzyme S and toxin A yields. The addition of NTA to the growth medium was found to be required for S production (Fig. 1 and 2) but not toxin A production (5, 15; and M. Bjorn, unpublished observation). NTA was required for S production regardless of the kind of growth medium used. NTA is a metal chelating agent which has been used by Callahan (5) to inhibit the activity of Pseudomonas proteases, which may be metalloenzymes (19). NTA at a concentration as low as 4 mM completely inhibited the protease activity of P. aeruginosa strain 388 (Fig. 2). Thus it would be tempting to conclude that NTA maximizes S yields by inhibiting the activity of proteases which in the absence of NTA destroy S. A comparison of the concentration of NTA required for maximum S yields (10 mM) and that (4 mM) required to inhibit detectable protease activity (Fig. 1 and 2) suggest that inhibition of protease activity is not the only mechanism by which NTA enhances S yields. This is supported by our observation that another protease inhibitor, $(NH_4)_2SO_4$ (16) could not substitute for NTA in

the growth medium. At a variety of concentrations of $(NH_4)_2SO_4$, in the absence of NTA, strain 388 produced no detectable exoenzyme S. Further evidence for the suggestion that inhibition of protease activity is not the only mechanism by which NTA enhances S yields was obtained by the use of an extracellular protease deficient mutant of strain 388. Despite its extracellular protease deficient phenotype, this mutant still requires 10 mM NTA in the medium in order for yields of exoenzyme S to be maximal (data not shown). Since NTA is a potent chelator it may be making one or more ions, which inhibit S yields, unavailable to the bacteria. Of those chelating agents examined only EDTA was able to substitute for NTA in the growth medium (Table 1). The concentration of EDTA or NTA which gave maximal S yields was 10 mM (Fig. 1 and Table 1). Although not identical, the stability constants of NTA and EDTA for a variety of metals are generally similar, whereas those of the other chelators tested (Table 1) generally differ from NTA and EDTA (17). The effect of NTA and EDTA is not only to reduce the iron concentration of the medium. In extensively deferrated medium, where the iron concentration was growth limiting, NTA or EDTA was still required for high S yields (data not shown). This result suggests that another metallic ion in addition to iron may regulate exoenzyme S yields. The exact mechanism by which NTA or EDTA enhance S yields is not yet known.

In addition to the requirement for NTA or EDTA in the growth medium, production of exoenzyme S differs from toxin A production in its response to the presence of crystalline BSA. Yields of S decreased in the presence of crystalline BSA (Fig. 6) whereas yields

of toxin A have been shown to increase in the presence of crystalline BSA (15). The mechanism whereby BSA inhibits S yields is not yet known. Whether other proteins will have a similar effect on S yields remains to be determined.

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Table 1. Effect of metal chelators on bacterial growth and exoenzyme S yields in cultures of *P. aeruginosa* 388[a]

Conc. of chelator	Tricine		Citrate		EDTA	
	Bacterial growth	ADPR incorporated	Bacterial growth	ADPR incorporated	Bacterial growth	ADPR incorporated
mM	(OD_{540})	(pmoles)	(OD_{540})	(pmoles)	(OD_{540})	(pmoles)
0.1	8.4	1.0	9.0	0.6	8.4	0.5
1.0	8.7	0.6	9.0	0.4	8.4	0.6
10.0	8.4	0.7	8.7	0.6	6.0	667
20.0	8.4	0.7	9.0	0.4	5.7	620

[a]Media were supplemented with 100 mM MSG and 1% glycerol. Cultures were incubated for 22 h with shaking at 32° C.

Table 2. Effect of glycerol on bacterial growth and exoenzyme S yields in cultures of *P. aeruginosa* 388[a]

Conc. of glycerol (%)	Bacterial growth (OD_{540})	ADPR incorporated (pmoles)
0	5.7	353
0.25	9.4	363
0.50	11.1	665
1.0	12.0	781
2.0	11.7	543

[a]Media were supplemented with 10 mM NTA and 100 MSG. Culture conditions were as stated in Table 1.

Table 3. The effect of temperature and aeration on bacterial growth and exoenzyme S yields in cultures of P. aeruginosa 388[a]

	Shaking cultures		Stationary cultures	
Temperature (C)	Bacterial growth (OD_{540})	ADPR incorporated (pmoles)	Bacterial growth (OD_{540})	ADPR incorporated (pmoles)
25	6.6	60	1.2	1
30	11.7	399	4.8	7
32	11.7	505	3.6	10
35	7.8	182	3.0	11
37	7.8	148	3.3	5

[a]Media were supplemented with 10 mM NTA, 100 mM MSG and 1% glycerol. The cultures were incubated for 22 h.

Table 4. Bacterial growth and exoenzyme S yields in various media[a]

Medium	Bacterial growth (OD_{540})	ADPR incorporated (pmoles)
TSB	15.6	15
TSBD	9.6	691
PP	10.8	526
PPD	6.9	371
Syncase	8.1	274
PGT	7.2	127

[a]Each medium was supplemented with 10 mM NTA, 100 mM MSG and 1% glycerol. Culture conditions were as stated in Table 1.

Table 5. Medium composition for the production of *P. aeruginosa* exoenzyme S[a]

Trypticase soy broth dialysate	30 g/liter H_2O
Nitrilotriacetic acid	10 mM
pH to	7.0
Monosodium glutamate	100 mM
Glycerol	1%

[a]The optimum culture conditions are: flask size to volume ratio of at least 10:1, adequate aeration and an incubation temperature of 32° C for 22 h.

Figure 1. Effect of various concentrations of NTA on the yields of exoenzyme S in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (△) bacterial growth as measured at 540 nm. Media were supplemented with 100 mM MSG and 1% glycerol. Cultures were incubated for 22 h with shaking at 32° C.

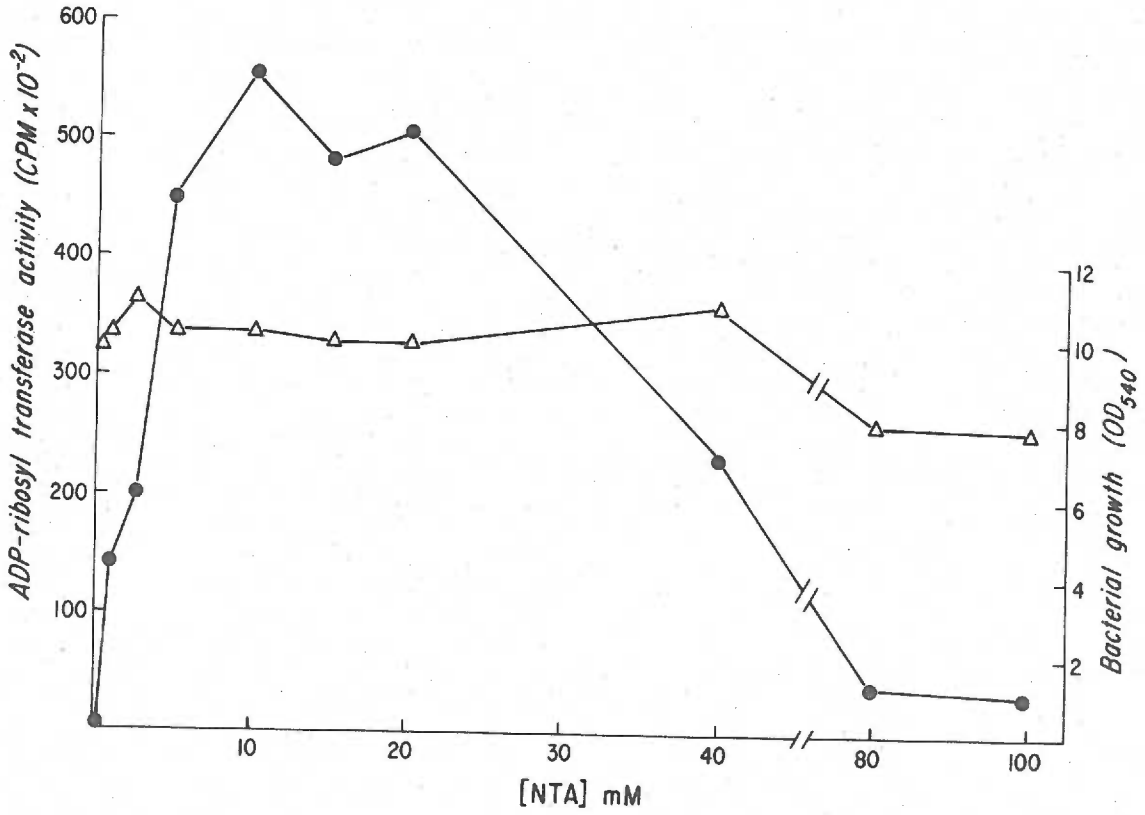

ADP-ribosyl transferase activity (CPM x 10⁻²)
600
500
400
300
200
100
10
20
30
40
80
100
[NTA] mM
12
10
8
6
4
2
Bacterial growth (OD540)

Figure 2. Effect of increasing concentrations of NTA on the yields of exoenzyme S and protease activity in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (□) protease activity in proteolytic units/ml. Media supplementation and culture conditions were as stated in Fig. 1.

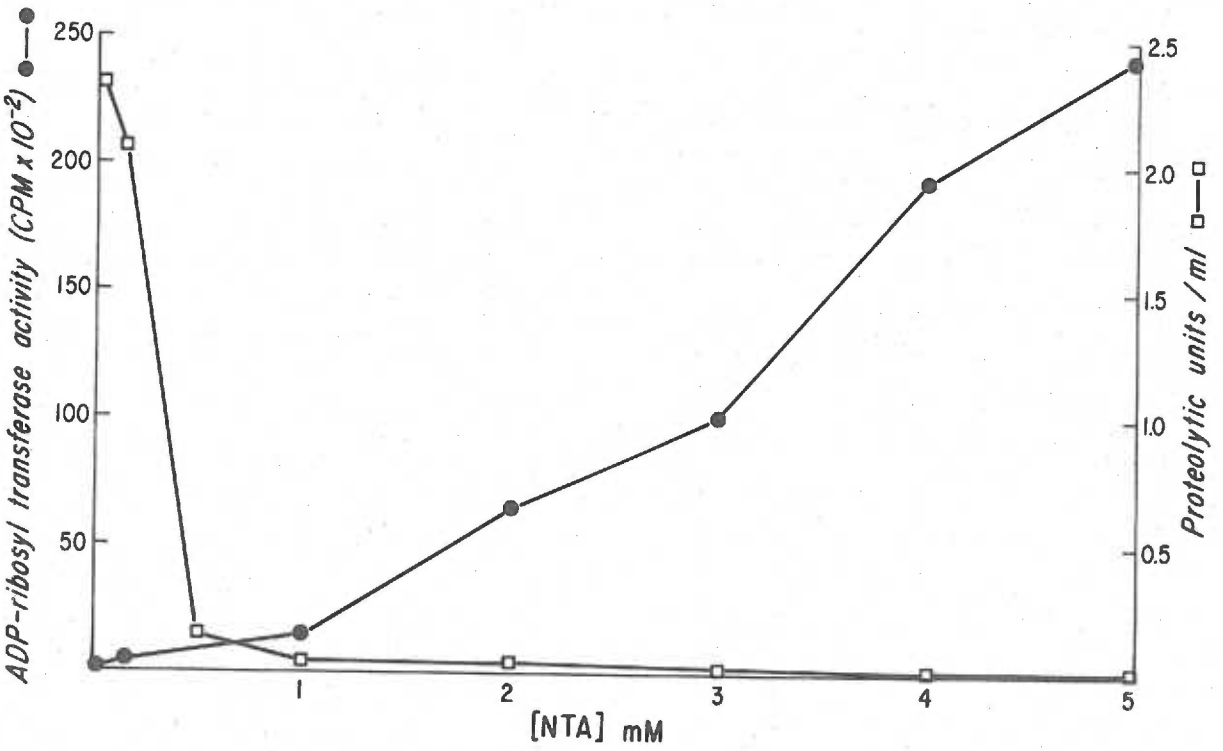

ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Proteolytic units / ml □—□
250
200
150
100
50
2.5
2.0
1.5
1.0
0.5
1
2
3
4
5
[NTA] mM

Figure 3. Effect of various concentrations of MSG on the yields of exoenzyme S in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (Δ) bacterial growth as measured at 540 nm. Media were supplemented with 10 mM NTA and 1% glycerol. Culture conditions were as stated in Fig. 1.

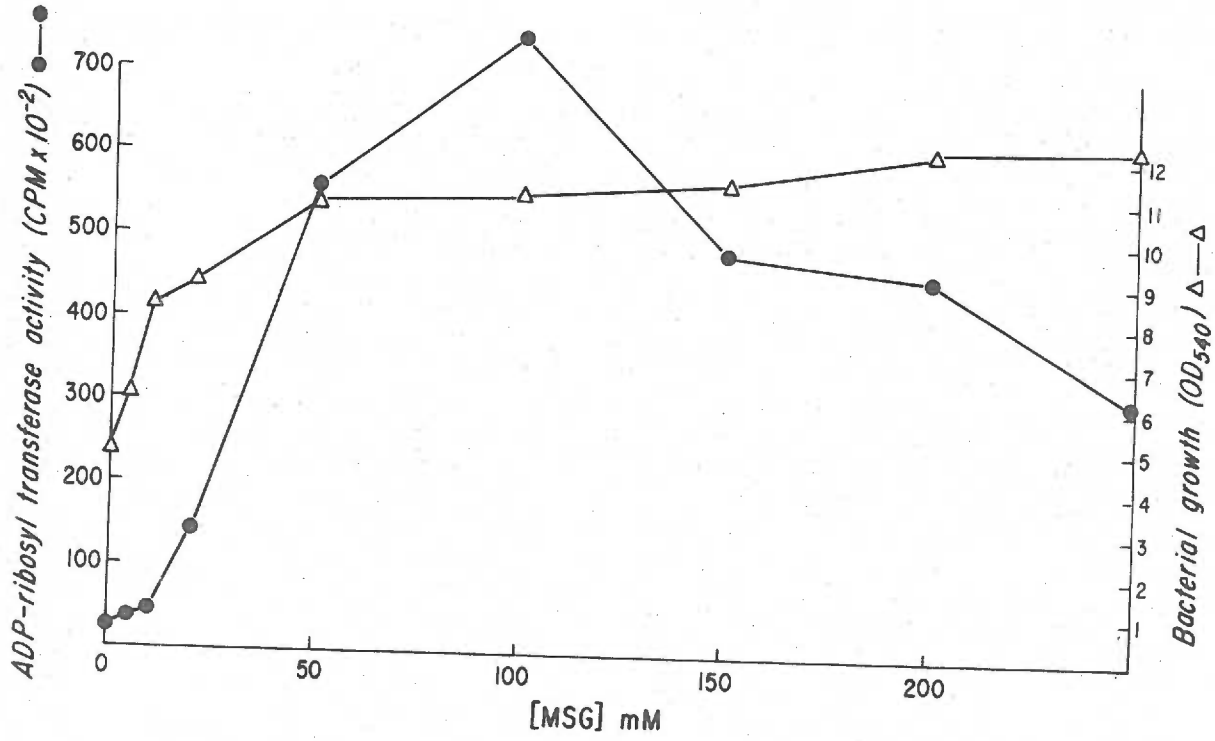

ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Bacterial growth (OD540) △—△
700
600
500
400
300
200
100
0
50
100
150
200
[MSG] mM
12
11
10
9
8
7
6
5
4
3
2
1

Figure 4. Effect of increasing concentrations of BSA on the yields of exoenzyme S in cultures of P. aeruginosa 388. S yields in the presence of A) impure (fraction V) BSA and B) crystalline BSA. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (Δ) bacterial growth as measured at 540 nm. Media supplementation was as stated in Fig. 3 and culture conditions as stated in Fig. 1.

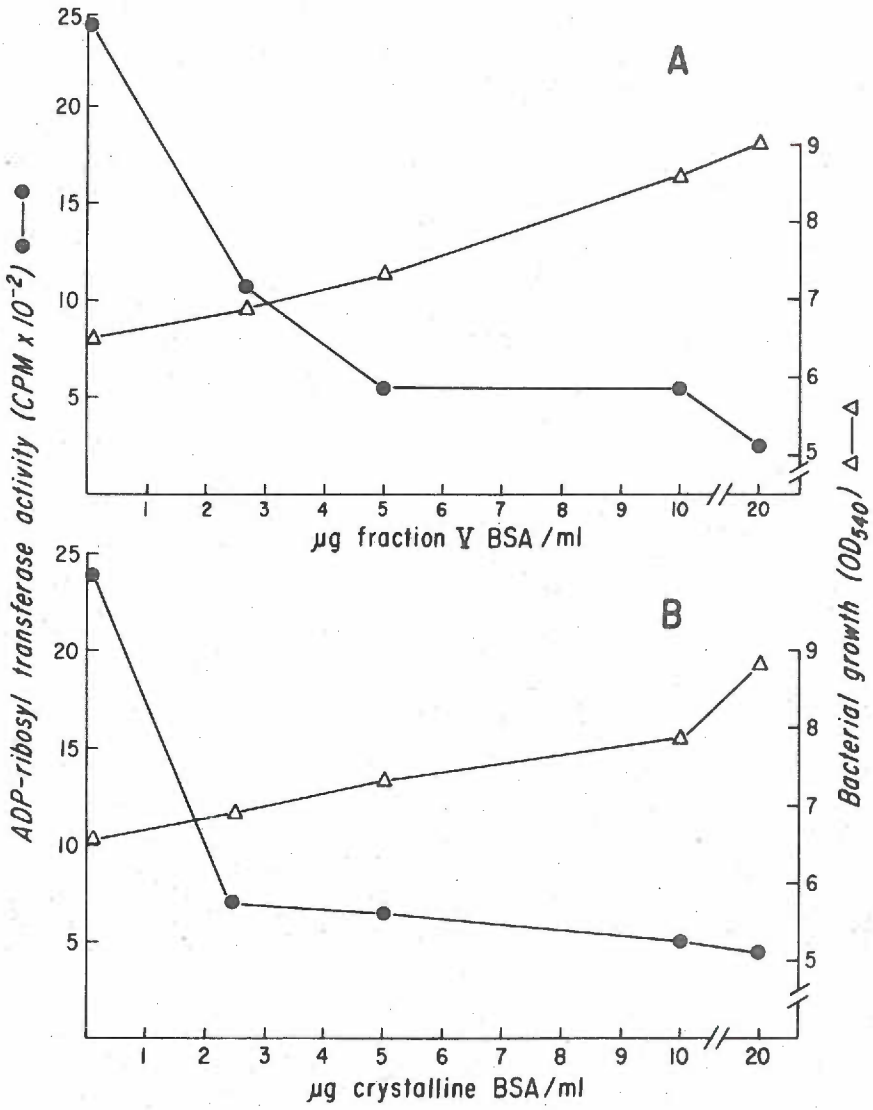

A
B
ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Bacterial growth (OD540) △—△
µg fraction V BSA / ml
µg crystalline BSA / ml
25
20
15
10
5
9
8
7
6
5
1 2 3 4 5 6 7 8 9 10 20

Figure 5. Effect of various concentrations of iron on the yields of exoenzyme S in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (Δ) bacterial growth as measured at 540 nm. Media were supplemented with 10 mM NTA, 100 mM MSG and 1% glycerol. Culture conditions were as stated in Fig. 1.

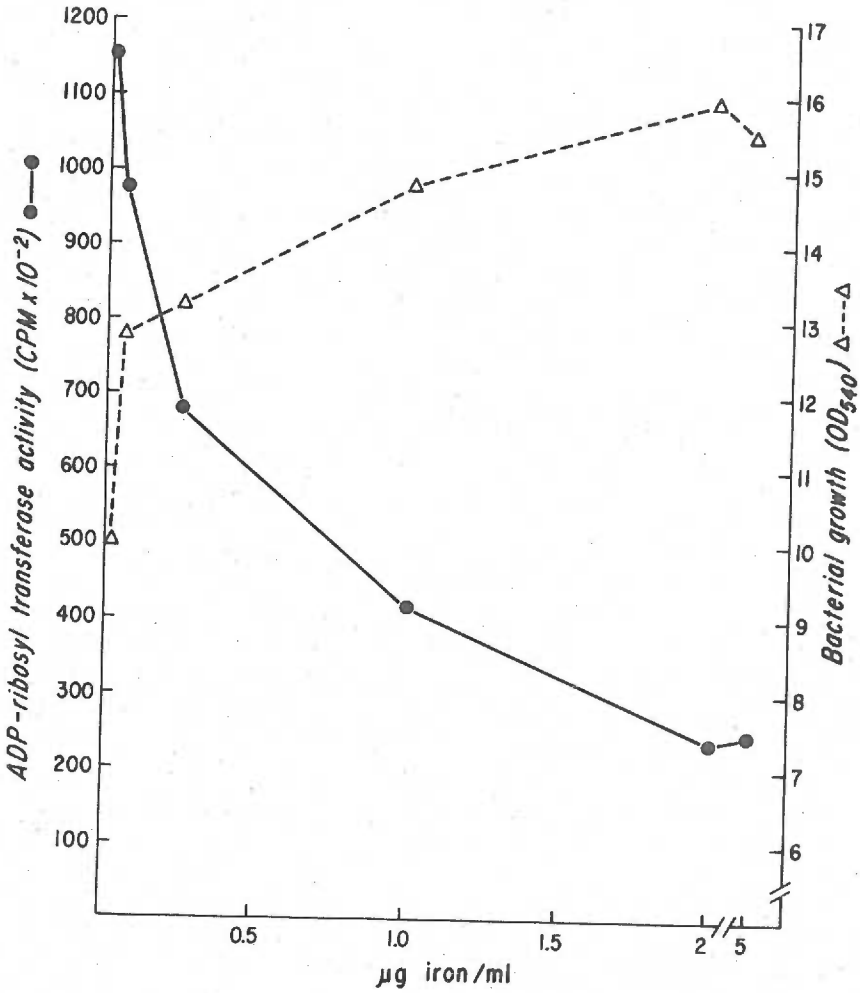
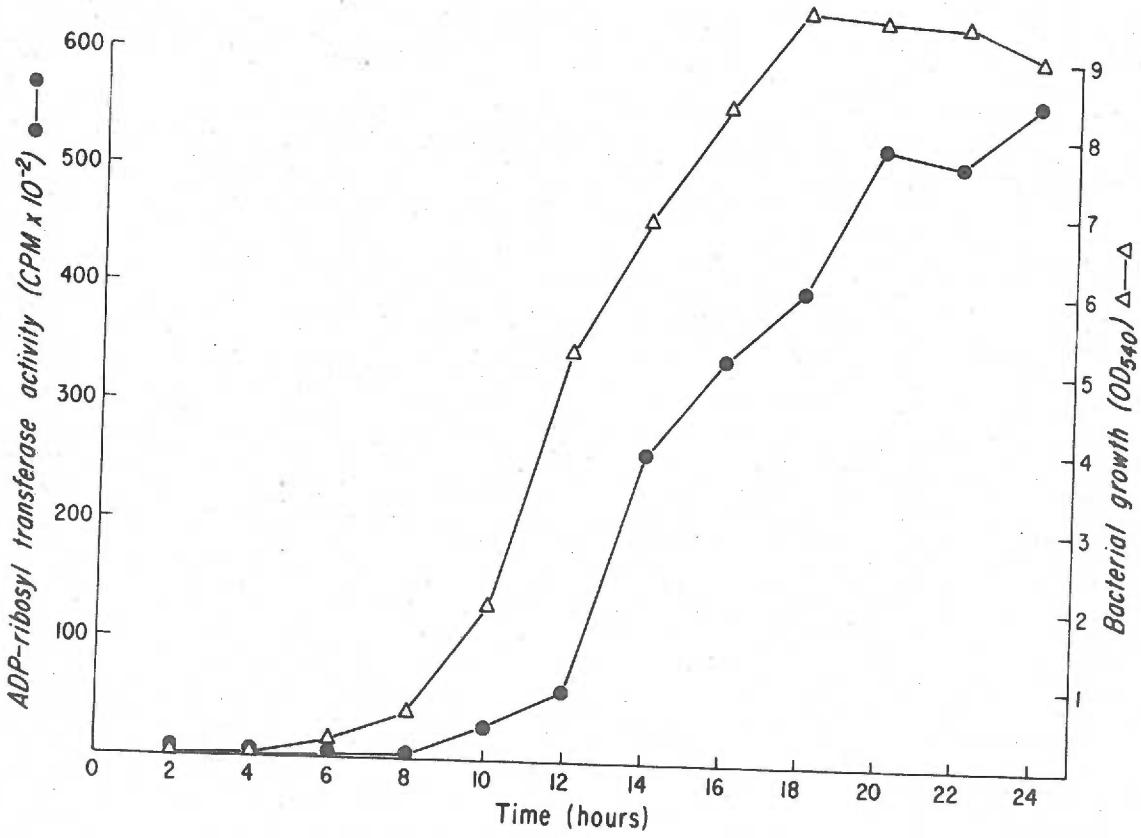

ADP-ribosyl transferase activity (CPM x 10⁻²)
Bacterial growth (OD540)
μg iron/ml
1200
1100
1000
900
800
700
600
500
400
300
200
100
17
16
15
14
13
12
11
10
9
8
7
6
0.5
1.0
1.5
2
5

Figure 6. Rate or release of exoenzyme S in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (Δ) bacterial growth as measured at 540 nm. Media were supplemented as stated in Fig. 5.


ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Bacterial growth (OD540) △—△
Time (hours)
600
500
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
22
24
9
8
7
6
5
4
3
2
1

V. Discussion and Summary

P. aeruginosa infections are a particularly severe problem in the compromised host such as cancer, burn and cystic fibrosis patients. The mechanisms responsible for the virulence of P. aeruginosa are not well understood. The organism produces a wide variety of extracellular products which may contribute to its pathogenicity (6). One of these, toxin A, is more toxic on a weight basis than any of the other products produced by P. aeruginosa (6). Toxin A inhibits protein synthesis in susceptible cells by catalyzing the transfer of the adenosine diphosphate ribosyl (ADP-ribosyl) moiety of nicotinamide adenine dinucleotide (NAD) onto elongation factor 2 (EF-2), in a manner identical to diphtheria toxin fragment A (3, 4). Despite the fact that its molecular mode of action is well understood, the role that toxin A plays in P. aeruginosa infections is not known. The toxin is lethal to experimental animals (6), produces a shock syndrome in dogs distinct from endotoxin shock (1), and is cytotoxic to a variety of tissue culture cells (7, 14). Toxin A has been detected in vivo in experimental animal models (11), and indirect evidence exists that toxin A is produced during P. aeruginosa infections of humans (10). In an experimental burned mouse model, the administration of A antitoxin has been shown to protect some mice and extend the survival times of others infected with toxin A producing strains of P. aeruginosa (9, 12).

Although the studies mentioned above indicate that toxin A is a potential virulence factor in P. aeruginosa infections, information

is still lacking regarding its precise role in disease. One of the necessary pieces of information that is missing is the prevalence of toxin A production among clinical isolates of P. aeruginosa. Methods have been developed to determine the incidence of toxin A production among clinical isolates of P. aeruginosa (paper 1 and Appendix B). The methods developed were (in increasing order of sensitivity) a modified Elek test, an ADP-ribosyl transferase assay and a reversed passive hemagglutination assay. Using combinations of these methods, at least 90% of P. aeruginosa isolates from urinary tract infections, septicemias and wounds were toxin A^+ (paper 1) while greater than 80% of P. aeruginosa strains from cystic fibrosis patients were toxin A^+ (Appendix B). None of the other Pseudomonas species tested besides P. aeruginosa produced detectable toxin A (paper 1). These data indicate that the toxin A gene is widespread in P. aeruginosa and add to the growing list of evidence supporting toxin A as a virulence factor.

The incidence of toxin A production among P. aeruginosa isolates from burn patients was also examined (Appendix D). Surprisingly, only 3 out of 22 burn isolates produced toxin A. However, the toxin A^- isolates were all of the same serotype and their antibiograms were similar, suggesting that these isolates were the same strain. Thus, the true incidence of toxin A production among different strains of P. aeruginosa may be much higher. Clearly, additional strains need to be examined in order to establish the incidence of toxin A^+ strains from burn patients. The most interesting suggstion resulting from

these experiments was that the burn patients infected with the toxin A^- isolates of P. aeruginosa had an unexpectantly benign clinical course (B. Minshew, personal communication). There are too many variables in this type of study to make solid conclusions, but the benign clinical course of burn patients infected with toxin A^- isolates of P. aeruginosa supports the hypothesis that toxin A plays a role in human infections. Perhaps the future prophylactic use of A toxoids and the therapeutic use of A antitoxin will provide protection against the effects of toxin A produced by P. aeruginosa in burn infections and other infections.

Understanding the physiology of toxin production can provide clues regarding the factors that regulate the production of toxins both in vitro and in vivo and are particularly important in terms of the economical production of a toxoid or antitoxin. Since the concentration of iron in the medium influences the final yields of both diphtheria toxin (DT) and Shigella dysenteriae type 1 toxin (Shiga toxin) (8, 13), the effect of this metal ion on toxin A yields was examined. In a manner similar to its effect on DT and Shiga toxin, the yields of toxin A were greatest in low iron medium and decreased as the iron concentration of the medium increased (paper 2). In vivo the levels of free iron in fluids and tissues are very low. Thus, if one was to extrapolate the in vitro situation to the in vivo environment, toxin A should be produced at optimal levels in the fluids and tissues of the host.

The inhibitory effect of iron was found not to be restricted to yields of toxin A. This effect has been extended to the yields of P. aeruginosa proteases and to a hemagglutinin (HA) (paper 3). A HA has not previously been reported for P. aeruginosa. The crude HA is non-dialysable, heat stable and resistant to trypsin and pronase treatment (paper 3). The biological role of the HA is not known at this time. Although the inhibitory effect of iron on proteases and HA yields was strain dependent, in one strain (WR-9), the initial appearance, rate of release and magnitude of inhibition of yields by iron were similar for toxin A, proteases and HA (paper 3). This suggests (at least in strain WR-9) that the yields of these extracellular products are regulated by one or more common mechanisms involving iron.

During the course of examining the incidence of toxin A production among clinical isolates of P. aeruginosa from burn patients, a new ADP-ribosyl transferase, exoenzyme S, was discovered (paper 4). Exoenzyme S differs from all known prokaryotic ADP-ribosyl transferases including P. aeruginosa toxin A. Exoenzyme S does not ADP-ribosylate EF-2, but rather another protein(s) present in extracts of eukaryotic cells. Exoenzyme S is more heat stable than toxin A and the enzymatic activity of S is partially destroyed by pretreament with urea and DTT, while such pretreatment potentiates the enzymatic activity of A (paper 4). Antisera against A and S have been prepared which specifically neutralize the enzymatic activity of the homologous, but not the heterologous, enzyme (paper 5). These antisera were used to show

that the enzymatic activity produced by strain 388 *in vivo* during an experimental animal infection was due to exoenzyme S. In addition, the presence of pre-existing S antibodies in some non-immunized animals suggests that some animals have previous exposure to exoenzyme S (paper 5). This latter finding suggests that exoenzyme S production is not rare among *P. aeruginosa*. Further studies are needed to identify the substrate modified by exoenzyme S, its toxicity and role in *P. aeruginosa* infections. These studies should be of particular interest since all prokaryotic ADP-ribosyl transferases identified so far are toxins or are involved in the regulation of macromolecular biosynthesis (2).

A detailed analysis of the factors that control the yields of exoenzyme S in cultures of *P. aeruginosa* strain 388 has led to the development of a medium and culture conditions which optimize the yields of S (paper 6). The medium and conditions are very similar to a medium found to optimize yields of toxin A (5, and paper 2). The media utilize TSBD as the base medium, include MSG and glycerol, and have a low concentration of iron. In addition, both A and S are produced in maximum yields at 32° C and in cultures that are well aerated. However, differences do exist in factors that regulate A and S yields (paper 6). The addition of crystalline BSA to the medium inhibits S yields, but reportedly (5) stimulates A yields. The addition of NTA or EDTA to the medium is mandatory for the production of maximum yields of S, but not A (paper 6). These compounds are potent chelating agents that inhibit protease activity. The mechanism(s) by

which NTA and EDTA maximize S yields, however, are not solely due to their inhibitory effect on proteases that might otherwise destroy S, but may also include the chelation of ions (in addition to iron) that specifically inhibit S yields (paper 6). The development of a suitable medium and culture conditions for optimizing S yields should aid in future studies on its purification and incidence of production among strains of P. aeruginosa.

Although this thesis has added new information regarding the production of P. aeruginosa ADP-ribosyl transferases, many unanswered questions remain. The precise role that toxin A plays in human disease is not yet known nor is the virulence potential of exoenzyme S understood. The mechanism(s) by which iron influences the yields of toxin A, exoenzyme S, proteases and HA are only speculative at this time. Finally, it is still unclear how the metal chelators NTA and EDTA stimulate the yields of exoenzyme S in cultures of P. aeruginosa. Answers to these questions will aid in understanding the biology of P. aeruginosa and may help in the future treatment and prophylaxis of P. aeruginosa infections.

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IV. Appendices

Appendix A: Attempt to demonstrate toxin A in the sputa of cystic fibrosis patients infected with Pseudomonas aeruginosa.

P. aeruginosa infections of the respiratory tract are a particularly severe problem in cystic fibrosis patients (4). The pathogenic mechanism(s) by which P. aeruginosa causes disease in the lungs of CF patients is not fully understood. One possibility is that toxin A, produced by organisms in the lungs of CF patients, contributes to the pathogenicity of P. aeruginosa. If this is the case, then it might be possible to detect toxin A in the sputa from CF patients infected with P. aeruginosa. A preliminary attempt was made to test this hypothesis.

Sputum samples from CF patients at the University of Oregon Health Sciences Center were collected in sterile containers and stored at -70° C. The highly viscous nature of the exocrine secretions from CF patients (1) necessitated the use of a mucolytic agent to digest the samples so that they could be tested in the ADP-ribosyl transferase assay. The mucolytic agent N-acetyl cysteine (Sigma) was utilized as described by W. Rourke (3). A 2% solution of N-acetyl cysteine (NAC) in PBS was prepared and the pH adjusted to 7.2 with NaOH. Equal volumes of the NAC solution and a sputum sample were mixed by vortexing and then incubated at 25° C for 15 min.

To detect toxin A, the digested sputa (10 µl) were pretreated with 10 µl of 8 M urea and 1% DTT and incubated at 25° C for 15 min. ADP-ribosyl transferase activity in the treated sputa was determined

in an enzyme assay as previously described (paper 2) using a 5 min incubation period at 25° C. As a positive control, 0.1 µg toxin A was added to a P. aeruginosa negative sputum sample. This sample was then digested with NAC, treated with urea and DTT and assayed for ADP-ribosyl transferase activity as described above. This sputum sample to which exogenous toxin A was added gave 525 counts per minute (cpm) in the ADP-ribosyl transferase assay. The same quantity of toxin A assayed without incubation in sputum and the NAC digestion gave 2,900 cpm (Table 1). Thus, the ADP-ribosyl transferase activity of toxin A was inhibited by the sputum, the NAC digestion or both. This may limit the sensitivity of the assay for detecting toxin A produced by P. aeruginosa in sputa.

The sputa from two separate groups of CF patients were assayed for ADP-ribosyl transferase activity. The first group included patients from whose sputa P. aeruginosa had been isolated, while Pseudomonas was not isolated from sputa of the second group of CF patients (Table 1). In group 1, two of the patients sputa yielded toxin A producing strains of P. aeruginosa as determined by the Elek test (paper 1), while the P. aeruginosa isolates from sputa of the other 3 patients were toxin A negative (Table 1). All sputa tested were negative in the ADP-ribosyl transferase assay (Table 1).

Thus, toxin A could not be detected in the sputa from CF patients when they were infected with toxin A producing strains of P. aeruginosa. This study was obviously limited by the small sample size. Another limitation was that the enzymatic activity of toxin A appeared to be inhibited by incubation in sputum or by the NAC digestion, or

both. Perhaps the use of the RPHA assay to detect toxin A in sputa would be advantageous, since it quantitates toxin A independent of its enzymatic activity (Appendix B). It would not be surprising if further studies using a larger sample size and more sensitive techniques demonstrate the presence of toxin A in the sputa of CF patients infected with P. aeruginosa. While we were unable to detect the presence of toxin A directly in sputa from CF patients, Klinger et al. (2) recently demonstrated the presence of toxin A antibody in sera from some CF patients.

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Table 1. Attempt at detecting toxin A in the sputa of cystic fibrosis patients infected with P. aeruginosa

Patient	P. aeruginosa		ADP-ribosyl transferase[a]
Group I	isolated	Elek test	activity in sputum (CPM)[b]
C.W.	+	-	33
M.M.	+	-	49
J.J.	+	-	50
J.D.	+	+	35
S.P.	+	+	34
Group II			
R.H.	-		35
J.R.	-		51
L.G.	-		39
R.K.	-		39
A.S.	-		36

[a]Background was 39 and was not subtracted from the samples.

[b]0.1 µg of toxin A gave 2,900 cpm's in the ADP-ribosyl transferase assay. When 0.1 µg of toxin A was incubated in sputum and treated with NAC, 525 cpm's were obtained in the transferase assay.

Appendix B. Incidence of toxin A production among Pseudomonas aeruginosa clinical isolates from cystic fibrosis patients.

The present study was undertaken to determine the incidence of toxin A production among isolates of P. aeruginosa from the respiratory tract of cystic fibrosis (CF) patients. The methods utilized to determine the incidence of toxin A production included a modified Elek test (paper 1), the direct detection of ADP-ribosyl transferase activity in cultural supernatants (paper 1) and a reversed passive hemagglutination assay (RPHA). The results presented in this study indicate that at least 80% of the CF clinical isolates of P. aeruginosa produced toxin A.

The Elek technique has previously been used as a convenient screening assay for identifying toxinogenic strains of P. aeruginosa (paper 1). In this study the Elek test was used to screen 130 isolates of P. aeruginosa from the respiratory tract of cystic fibrosis patients. Sixty-one (47%) of the 130 isolates were toxin A positive as determined by this technique (Table 1).

In a previous study (paper 1) there was a strong positive correlation between an Elek positive isolate of P. aeruginosa and its ability to produce extracellular ADP-ribosyl transferase activity. Therefore, only those CF isolates that were negative in the Elek test were examined for toxin A by ADP-ribosyl transferase activity. Of the 69 Elek negative strains examined, 24 produced toxin A as determined by ADP-ribosyl transferase activity. It was necessary to establish that the ADP-ribosyl transferase positive strains were toxin

A producers and that the enzymatic activity was due to toxin A and not the other known P. aeruginosa ADP-ribosyl transferase, exoenzyme S (paper 4). To test this, the ability of A antitoxin to neutralize the enzymatic activity of 10 of the isolates was examined. The ADP-ribosyl transferase activities of the 10 isolates were neutralized by A antitoxin (Table 2). Thus the Elek negative, ADP-ribosyl transferase positive isolates were toxin A positive. If a toxin positive strain is defined as giving a positive Elek or as positive in the ADP-ribosyl transferase assay, then 65% (85/130) of the P. aeruginosa isolates from CF patients were toxin A positive (Table 1).

In order to further evaluate the 45 isolates that were negative in both the Elek test and the ADP-ribosyl transferase assay, a reversed passive hemagglutination assay was developed. The following is a description of the assay. Antitoxin A gamma-globulin was obtained from rabbit immune serum as previously described (4). The immunoglobulin fraction was further purified on a solid-phase immunoabsorbent (Sepharose 4B) with covalently linked purified toxin A utilizing a previously described method (paper 5). The immunoabsorbent purified antitoxin A IgG contained 0.5 mg protein per ml.

The RPHA assay for toxin A was developed using modifications of the procedure of Holmes et al. (3) which they used to quantitate cholera toxin. A stock solution of Bis-diazotized benzidine (BDB) was prepared and stored exactly as described (1). Formalinized sheep erythrocytes were prepared (2) and stored at 4° C. The sensitizing mixture varied from batch to batch of BDB and had to be determined

empirically whenever a new stock solution of BDB was used. A representative sensitizing mixture consisted of: 1.8 ml buffer A (0.082 M NaCl, 0.043 M $Na_2HPO_4 \cdot 2H_2O$, 0.0107 M KH_2PO_4, pH 7.4), 0.240 ml of 10% formalinized erythrocytes, 0.100 ml of purified anti-A immunoglobulin and 0.240 ml of a 1:10 dilution of BDB stock solution in buffer A. The cells were sensitized by incubation of the above mixture for 10 min at 37° C with gentle agitation (3). The sensitized erythrocytes were washed as described by Holmes et al. (3) and suspended to 0.2% in diluent (buffer A containing 1% crystalline BSA and 0.01% merthiolate). The sensitized erythrocytes were used on the same day that they were prepared.

RPHA assays were performed in plastic microtiter trays with V-bottom wells (linbro Chemical Co., Inc.) in 100 µl reaction mixtures containing serial two-fold dilutions of the test samples (starting at 1:2), antitoxin sensitized erythrocytes at a final concentration of 0.1%, and diluent. Serial dilutions of purified toxin A provided a positive control for agglutination. On most days the RPHA assay was capable of detecting quantities of toxin A as low as 100 pg (2 ng/ml). Negative controls included both antitoxin-sensitized SRBC without added toxin or supernatant and unsensitized erythrocytes with serial dilutions of toxin or supernatant. This latter negative control was particularly critical since some isolates of P. aeruginosa produce a hemagglutinin (HA; paper 3) which interferes with the RPHA assay.

Nineteen of the 45 Elek negative, ADP-ribosyl transferase negative isolates produced sufficient quantitites of HA to interfere with the RPHA assay. Of the remaining 26 isolates, 19 produced detectable

quantities of toxin A as determined by the RPHA assay. These isolates produced very small amounts of toxin A ($\leq$0.1 μg/ml) consistent with the observation that they were negative in the Elek test and ADP-ribosyl transferase assays. No detectable toxin A ($\leq$0.004 μg/ml) was found in the supernatants of 7 of the isolates as measured by the RPHA assay.

If a toxin A positive strain is redefined as being positive in either the Elek test, ADP-ribosyl transferase assay or RPHA assay, then 80% (104/130) of the P. aeruginosa isolates from cystic fibrosis were toxin A positive (Table 1). The true incidence of toxinogenesis may be much higher than 80% since 19 of the isolates could not be evaluated by the most sensitive of the assays utilized (RPHA) because they produced interfering HA activity. However, it does appear as if a larger percentage of the CF isolates produced smaller quantities of toxin A, compared to P. aeruginosa isolates from patients with septicemias, wounds, and urinary tract infections (paper 1). In that study (paper 1), 90% of the isolates produced toxin A as detected by the Elek test and ADP-ribosyl transferase activity, while in the present study only 65% of the CF isolates produced toxin A as detected by these same techniques (Table 1).

The isolates for this study were obtained from two separate CF clinics, one at the University of Oregon Health Sciences Center, Portland, Oregon, and the other at the Ohio State University, Columbus, Ohio. The incidence of A toxinogenesis was the same in the isolates from the two clinics (Portland: 21/38 [79%]; Columbus: 74/92 [80%]). Thus the toxin A gene is widespread among isolates of P. aeruginosa

(see also paper 1).

An unusual characteristic of P. aeruginosa isolates from cystic fibrosis patients is that they are often mucoid in colonial morphology, while isolates of P. aeruginosa from other patient populations are rarely mucoid (5). In this study, 45% of the clinical isolates from CF patients were mucoid. The incidence of toxin A production was approximately the same in the mucoid and non-mucoid isolates (80%).

As previously described in detail in this thesis (see Literature Review), P. aeruginosa toxin A is a potential virulence factor. The role that toxin A plays in Pseudomonas respiratory tract infections of cystic fibrosis patients is not known. This report is a first step in evaluating this role. Since most isolates of P. aeruginosa from cystic fibrosis patients produce toxin A, it is possible that the production of this toxin in vivo contributes to the virulence of P. aeruginosa in CF patients. Further studies are needed to evaluate this hypothesis.

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Table 1. Incidence of toxin A production among clinical isolates of Pseudomonas aeruginosa from cystic fibrosis patients

	No. positive/tested[a]	% positive
Elek test	61/130	47
ADP-ribosyl transferase activity	85/130	65
RPHA	104/130	80

[a]The assumptions were made that an isolate that was Elek positive would be positive in both the ADP-ribosyl transferase assay (paper 1) and the RPHA and an isolate that was positive in the ADP-ribosyl transferase assay would be positive in the RPHA (Appendix C).

Table 2. Neutralization of the ADP-ribosyl transferase activity from *P. aeruginosa* cystic fibrosis isolates by A antitoxin[a]

Isolate	ADP-ribosyl transferase activity (CPM)[b]	
	NRS[c]	A antitoxin
8446_I	136	0
P.L.	115	10
Ku	120	18
Ra	109	4
7768	87	0
MH620	188	10
MC	213	0
TF	164	3
R2985	75	0
LW	92	0

[a]Neutralization was performed as described in paper 5.

[b]Background was subtracted from all samples.

[c]NRS is normal rabbit serum.

Appendix C. Comparison of a reversed passive hemagglutination assay with ADP-ribosyl transferase activity for quantitating Pseudomonas aeruginosa toxin A.

Two distinct assay methods have been developed to quantitate toxin A in crude culture supernatants of P. aeruginosa. One of the methods was based on enzymatic activity (ADP-ribosyl transferase) and the other on immunological reactivity (reversed passive hemagglutination assay; RPHA). In this study, the quantitation of toxin A by the two methods was compared. P. aeruginosa strain WR-9 was grown in deferrated TSBD medium supplemented with increasing concentrations of iron ($FeSO_4 \cdot 7H_2O$). After 22 h of growth the supernatants were obtained and dialysed for 18 h against 0.01 M Tris·HCl buffer, pH 8.0 at 4° C. The quantity of toxin A as a function of ADP-ribosyl transferase activity was determined as described in an earlier section of this thesis (paper 3) based on standard curves of the enzymatic activity of activated (urea + DTT-treated) pure toxin A. Toxin A as quantitated by the RPHA assay was determined as described in Appendix B.

The levels of toxin A in crude supernatants of strain WR-9 were shown to be remarkably similar by either method (Table 1). The major problem with the RPHA assay is that the location of the endpoint is based on a subjective judgment by the experimenter. In assays such as the RPHA assay, it is usually stated that there is a one well margin of error on either side of the chosen endpoint. Therefore, we have shown the range of values for the RPHA assay in Table 1. Toxin A levels based on ADP-ribosyl transferase activity is a more precise technique.

Triplicate samples usually do not vary by more than 10% (Table 1).

The major advantage of the RPHA assay is its sensitivity. This method has been used in our laboratory to measure toxin A levels as low as 100 pg (2 ng/ml). The maximum sensitivity of the ADP-ribosyl transferase assay in our laboratory has been 50 ng/ml (paper 3). Thus, although the RPHA is less precise than the ADP-ribosyl transferase assay, it is ∿25 times more sensitive. A practical limiting factor of the RPHA assay for quantitating toxin A in crude supernatnats of P. aeruginosa is the presence of an interfering hemagglutinin (paper 3 and Appendix B). Depending on the strain, the hemagglutinin can make the RPHA assay useless for detecting small amounts of toxin A in crude supernatant fluids of P. aeruginosa strains.

In summary, this study has shown that both the ADP-ribosyl transferase and RPHA assays are reliable methods for quantitating toxin A. The major advantage of the RPHA assay for quantitating toxin A is its greater sensitivity while the ADP-ribosyl transferase assay is more precise.

Table 1. Comparison of a reversed passive hemagglutination assay and the ADP-ribosyl transferase assay for quantitating toxin A in supernatants of *P. aeruginosa* strain WR-9

Iron in medium µg/ml	ADP-ribosyl transferase assay µg toxin A/ml[a]	RPHA assay µg toxin A/ml[b]
0.04	3.4 (3.1-3.7)	5.1 (2.6-10.2)
0.20	2.1 (1.9-2.3)	1.9 (0.95-3.8)
0.50	0.4 (0.3-0.5)	0.12 (0.06-0.24)
1.5	0.07 (0.06-0.085)	0.08 (0.04-0.16)
5.0	0.08 (0.065-0.09)	0.06 (0.03-0.12)

[a]Triplicate assays of the same sample usually do not vary by more than 10% in the ADP-ribosyl transferase assay.

[b]The range of toxin A levels (µg/ml) are in parenthesis based on a one well margin of error on either side of the true endpoint.

Appendix D. Toxin A and exoenzyme S production by Pseudomonas aeruginosa strains isolated from human burn wounds.

Pseudomonas infections were first recognized as a significant problem in burn patients in 1961 by Tumbusch et al. (4). Before and during the early 1940's, Streptococcus was the most significant pathogen infecting burn patients, but with the development and use of penicillin, Staphylococcus became the predominant life-threatening microorganism in the 1950's (3). Since then, the use of antimicrobials against Staphylococcus has been associated with the emergence of Pseudomonas as the bacterial species most often associated with fatal infections of the burn wound (3, 5). In patients with burns over 30% of their total body surfaces, the likelihood of clinically significant infection with P. aeruginosa is great (1, 5). In the absence of topical therapy, 70% of burn wounds are colonized by Pseudomonas by the end of the third week after the burn (1).

Two of the major reasons that Pseudomonas is such a threat to burn patients is its resistance to many of the commonly used antibiotics (1, 5) and its ability to persist in the hospital environment where other pathogens are readily killed (1, 5). However, elucidation of specific virulence factors which contribute to the pathogenicity of P. aeruginosa in burn patients has not been well studied. As previously described in this thesis, two P. aeruginosa ADP-ribosyl transferases, toxin A and exoenzyme S, have the potential to be virulence factors. The present study was an attempt to demonstrate the production of toxin A and exoenzyme S by P. aeruginosa isolates from burn patients.

P. aeruginosa isolates from burn patients were provided by Dr. B. Minshew, University of Washington, Seattle. The isolates were obtained from the burn unit at Harborview Hospital, Seattle. The antibiograms of the isolates were performed by Dr. Minshew. The P. aeruginosa isolates were serotyped using the 7 immunotyping sera of Fisher et al. (2).

The ability of the strains to produce toxin A was determined by a modified Elek test (paper 1) and by ADP-ribosyl transferase activity in 22 h supernatants from cultures grown in TSBD medium as described in paper 2. The ability to produce exoenzyme S was determined by ADP-ribosyl transferase activity in cultures grown in TSBD medium containing 10 mM NTA as described in paper 4. Based on previous studies (paper 6), an isolate was identified as exoenzyme S^+ if it produced high amounts of ADP-ribosyl transferase activity in medium supplemented with NTA, but low levels of ADP-ribosyl transferase activity in medium without NTA, and if the enzymatic activity was partially destroyed by urea and DTT pretreatment (paper 4).

Only 3 of the 22 P. aeruginosa isolates from burn patients were able to produce detectable quantities of toxin A or exoenzyme S (Table 1). These 3 isolates differed from the other 19 isolates in that they were not serotype 2. It is interesting that all 3 isolates that produced toxin A also produced exoenzyme S (Table 1). It is not yet known if this represents a significant relationship or if it was entirely fortuitous. Two of the 3 A^+, S^+ isolates shared the same serotype and antibiogram, suggesting that they were the same strain.

All of the serotype 2 isolates were negative for both toxin A and exoenzyme S (Table 1). Those serotype 2 isolates that were tested had identical or similar antibiograms (Table 1), suggesting that they represent the same strain. This strain has been the predominant P. aeruginosa organism isolated from burn patients at Harborview Medical Center during the past 2 years. Burn patients infected with this strain generally have had unexpectedly benign clinical courses (B. Minshew, personal communication). As expected, skin extracts and sera obtained from patients with the A^-, S^- P. aeruginosa strain (serotype 2) were negative for exogenous ADP-ribosyl transferase activity. Unfortunately, skin extracts and sera from the patients infected with the A^+, S^+ isolates were not available.

From this limited study, the true incidence of toxin A and exoenzyme S production among burn isolates of P. aeruginosa could not be ascertained. The most intersting aspect of this study was the predominance of one P. aeruginosa strain as the organism most frequently colonizing burn wounds in the patients from a single burn center over a 2 year period. Generally, burn patients infected with this strain had an unexpectedly benign clinical course. Further studies are needed to determine the incidence and role of exoenzyme S and toxin A in P. aeruginosa burn infections of humans.

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Table 1. Characteristics of *P. aeruginosa* isolates from burn patients

	Serotype	Toxin A	Exoenzyme S	Ampicillin	Cephalothin	Tetracycline	Chloramphenicol	Kanamycin	Gentamicin	Tobramycin	Carbenicillin	Amikacin
Patient #1												
Culture no.												
152-050 R. thigh	2	-	-	R	R	R	R	R	I	S	S	S
152-053 R. groin	2	-	-	R	R	R	R	R	I	S	S	S
156-051 L. thigh	2	-	-	R	R	R	R	R	I	S	S	I
156-045 L. shoulder	2	-	-	R	R	R	R	R	I	S	S	S
Patient #2												
177-027 L. leg	2	-	-	R	R	R	R	R	I	S	S	S
177-025 L. flank	2	-	-	R	R	R	R	R	I	S	S	I
Patient #3												
240010 sputum	2	-	-	R	R	R	R	R	R	I	R	R
242029 R. flank	2	-	-	R	R	R	R	R	R	I	R	R
241058 swanz ganz tip	2	-	-	R	R	R	R	R	I	S	S	S
240062 shoulder	2	-	-	R	R	R	R	R	R	I	R	R
240063 #1 L. breast	2	-	-	R	R	R	R	R	I	S	S	S
240063 #2 L. breast	2	-	-	R	R	R	R	R	R	I	R	R
Patient #4												
181025A R. flank	2	-	-	R	R	R	R	R	R	S	S	S
181025B R. flank	2	-	-	R	R	R	R	R	R	R	R	R
192079 Blood	2	-	-	R	R	R	R	R	I	R	R	R
Patient #5												
209050 unknown	2	-	-	R	R	R	R	R	S	S	S	S
Patient #6												
5043 unknown	3	+	+	Not determined								

Table 1 (continued)

	Serotype	Toxin A	Exoenzyme S	Ampicillin	Cephalothin	Tetracycline	Chloramphenicol	Kanamycin	Gentamicin	Tobramycin	Carbenicillin	Amikacin
Patient #7												
57 unknown	1	+	+	R	R	R	R	R	I	S	R	R
Patient #8												
262 unknown	1	+	+	R	R	R	R	R	I	S	R	R
Patient #9												
100A unknown	2	-	-	Not determined								
Patient #10												
101 C unknown	2	-	-	Not determined								
Patient #11												
102B unknown	2	-	-	Not determined								

Appendix E: The effect of the ferric ion on Pseudomonas aeruginosa toxin A.

In previous reports (papers 2 and 3), it was shown that the concentration of iron in the medium influenced the yields of toxin A in cultures of all A toxinogenic strains tested. Those studies were performed using the ferrous (Fe^{++}) form of iron. It was of interest to determine if the addition of the ferric (Fe^{+++}) ion to the culture medium would result in similar effects on A yields.

TSBD medium was deferrated with calcium chloride as described in paper 2. The deferrated medium contained 0.025 µg Fe/ml. Standard sterile solutions of the ferric ion in the form of $Fe_2(SO_4)_3 \cdot 6H_2O$ were added to the medium. Other than the addition of Fe^{++} in place of Fe^{++} the culture conditions were as described in paper 2. The effect of varying the concentrations of Fe^{++} on the yields of toxin A in cultures of P. aeruginosa strain PA-103 was examined.

As expected, bacterial growth in the 22 h cultures increased with the addition of the ferric ion to the deferrated medium (Fig. 1). The final yield of bacteria as measured by OD_{540} increased approximately two-fold in the medium to which 10 µg Fe/ml was added as compared to the deferrated medium containing 0.025 µg iron/ml (Fig. 1).

The extracellular yields of toxin A as determined by ADP-ribosyl transferase activity were decreased as the ferric ion concentration of the medium increased (Fig. 1). The yield of toxin A was

measurably decreased by the addition of as little as 0.3 µg Fe^{+++}/ml. Toxin A yields in medium supplemented with 1.5 µg Fe^{+++}/ml were decreased by approximately 90% as compared to yields in the deferrated medium. The magnitude of the inhibition of A yields began to level off at concentrations of ferric ion greater than 1.5 µg/ml (Fig. 1).

These results show the ferric ion, as well as ferrous ion (papers 2 and 3), inhibits the yields of toxin A. The magnitude of the inhibition was very similar for the two forms of iron as can be seen by comparing Fig. 1 with Fig. 2, paper 2 of this thesis.

It is possible that only one form of iron (Fe^{++} or Fe^{+++}) is responsible for the inhibition of A yields. This would imply the conversion of the non-inhibitory form of iron into the inhibitory form. Edwards and Seamer (1) have presented evidence that only the ferrous form of iron inhibits diphtheria toxin yields. Their data suggest that ferric ions are rapidly converted into the ferrous form of iron before exerting its inhibitory effect on diphtheria toxin yields. Further studies are needed to determine if a similar conversion occurs in P. aeruginosa.

References

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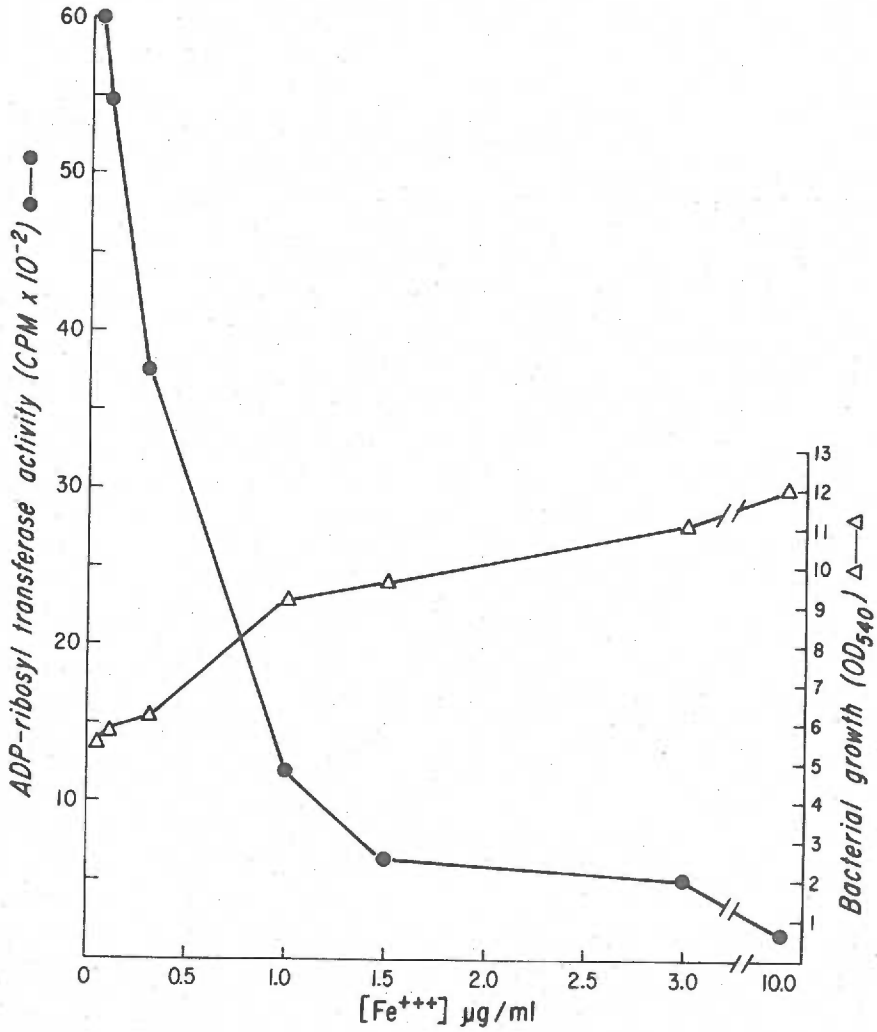
Figure 1. The effect of iron (Fe^{+++}) on bacterial growth and toxin A yields in P. aeruginosa strain PA-103. cpm x 10^{-2} is the acid-insoluble radioactive counts per min in the ADP-ribosyl transferase assay of activated (urea + DTT-treated) samples. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (Δ) bacterial growth as measured by optical density at 540 nm.


ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Bacterial growth (OD540) △—△
[Fe+++] µg/ml
60
50
40
30
20
10
13
12
11
10
9
8
7
6
5
4
3
2
1
0
0.5
1.0
1.5
2.0
2.5
3.0
10.0

Appendix F. Rate of toxin A release by Pseudomonas aeruginosa WR-9; detection and quantitation of toxin A by a reversed passive hemagglutination assay.

The ADP-ribosyl transferase assay has been used to examine the initial appearance and the rate of release of toxin A in cultures of P. aeruginosa strain PA-103 and WR-9 (papers 2 and 3). In the present study, the RPHA assay (Appendix B) was utilized to examine the rate of release of toxin A in cultures of WR-9 grown in low (0.05 µg/ml) and high (5.0 µg/ml) iron medium.

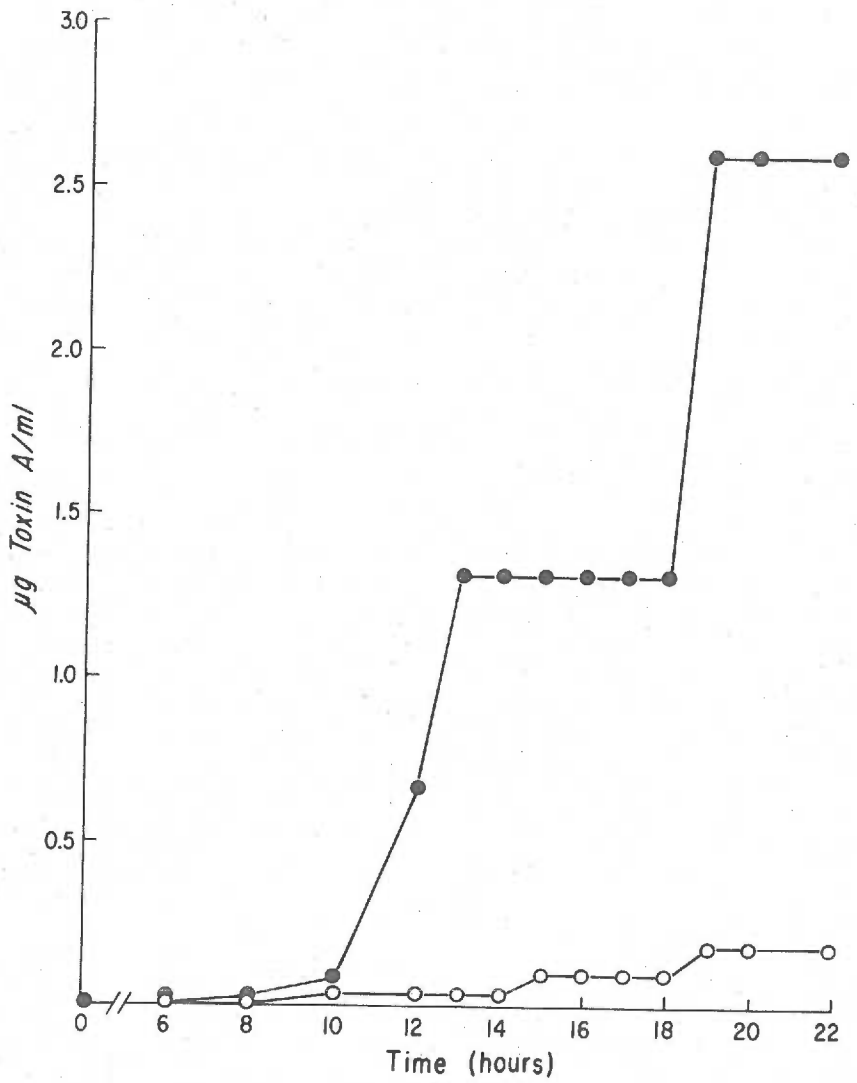
Although WR-9 does produce a hemagglutinin (HA) (paper 3), there was no interference with the RPHA assay since the HA titer was always lower than the RPHA titer. The initial appearance of toxin A in both the high and low iron medium was at 8 h (Fig. 1). The yields of toxin A in the low iron medium increased between 10 h and 18 h and then leveled off at 2.6 µg toxin A/ml. In the high iron medium the yields of toxin A gradually increased through 19 h to 0.18 µg toxin A/ml, following which no further increase was seen (Fig. 1).

The step-like appearance of Fig. 1 was due to the relative imprecision of the RPHA assay. Two-fold serial dilutions of the supernatants were made. For example, the quantity of toxin A in low iron medium at 20 h was read as 1.3 µg/ml or 2.6 µg/ml. Intermediate values could not be obtained using the two-fold dilution series.

By comparing Fig. 1 of this appendix with Fig. 1, paper 3, it can be seen that the rate of release of toxin A in cultures of WR-9 was

similar as determined by the RPHA assay (immunologically) and by ADP-ribosyl transferase activity. The major difference in these data was that the RPHA assay was capable of detecting toxin A earlier, which is consistent with the greater sensitivity of the RPHA assay as compared to that of the ADP-ribosyl transferase assay (Appendix B). Thus, the rate of toxin A release was similar as measured either immunologically or enzymatically.

Figure 1. Rate of toxin A release in cultures of P. aeruginosa WR-9 grown in low (0.05 µg/ml) and high (5.0 µg/ml) iron medium as determined by a reversed passive hemagglutination assay. Symbols: rate of release of toxin A in low iron (●) and in high iron (Δ) medium.


3.0
2.5
2.0
1.5
1.0
0.5
µg Toxin A/ml
0
6
8
10
12
14
16
18
20
22
Time (hours)

Appendix G. Effect of various metal ions on P. aeruginosa toxin A yields.

To date, iron is the only metal ion that has been shown to dramatically affect the yields of diphtheria toxin (1, 2), Shigella dysenteriae type 1 toxin (3) and P. aeruginosa toxin A (papers 2 and 3). In the case of Pseudomonas toxin A, iron is the only metal ion whose effect on A yields has been examined (papers 2 and 3). In this study, the effect of 6 metal ions on toxin A yields were compared to the effect of iron. The ions included 3 transition elements (cobalt, copper and zinc) and 3 divalent cations from group II of the periodic table (magnesium, calcium and barium). The ions were added to deferrated TSBD medium (paper 2) in the form indicated in Table 1. The effects of adding 1 µg/ml and 5 µg/ml of the appropriate ion on bacterial growth and toxin A yields of 22 h cultures were examined. The culture conditions were as described in paper 2 of this thesis.

Bacterial growth in the deferrated medium was 9.3 OD_{540} units. The only ion tested that caused a striking increase in bacterial growth was iron (Table 1). Growth in the presence of 1 µg/ml or 5 µg/ml of the other ions was not markedly affected (Table 1).

As previously described (papers 2 and 3) toxin A yields were decreased by increasing concentrations of iron (Table 1). Most of the other ions caused a slight increase in toxin A yields and none of these ions caused a substantial decrease in A yields (Table 1). Thus it appears that of the metal ions tested, only iron had a

striking effect on bacterial growth and on the extracellular yields of toxin A. Only seven metals were tested and it is possible that metals not included in this study will be found to dramatically affect the yields of toxin A.

The medium used in this experiment was deferrated by the calcium chloride method as described in paper 2 and the concentration of iron in the deferrated medium was 0.05 µg/ml. No attempt was made to remove the other metals from the medium, although some of the metals should be absent or in very minute quantities in TSBD medium. It is also possible that the deferration procedure non-specifically removed significant amounts of some metals. The concentrations of four of the metals in the deferrated medium were determined by Jack Lile of Dr. Iglewski's laboratory using flame atomic absorption spectroscopy. These concentrations were: Mg, 14.2 µg/ml; Mn, 0.05 µg/ml; Zn, 1.27 µg/ml; and Cu, <0.05 µg/ml. Using this method the concentration of iron in the deferrated medium was less than 0.05 µg/ml. Although the precise concentration of Ca was not determined, it was most probably high since in the deferration procedure, a large quantity of $CaCl_2$ was added.

In conclusion, the inhibitory effect of iron on toxin A yields does not appear to be a general phenomenon shared by all metals. Of the seven metals tested in this study, only iron dramatically affected bacterial growth (increase) and toxin A yields (decrease). Regulatory mechanisms in P. aeruginosa that control A yields appear to be mediated specifically by iron.

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Table 1. Effect of metals on the growth of *P. aeruginosa* strain PA-103

Metal	Added in form of:	Bacterial growth (OD_{540}) 0 µg/ml	1 µg/ml	5 µg/ml
Ba	$BaCl_2 \cdot 2H_2O$	9.3	9.5	9.0
Ca	$CaCl_2 \cdot 2H_2O$	"	9.1	9.1
Cu	$CuSO_4 \cdot 5H_2O$	"	10.0	9.8
Co	$Co(NO_3)_2 \cdot 6H_2O$	"	10.0	10.0
Fe	$FeSO_4 \cdot 7H_2O$	"	13.0	15.0
Mg	$MgSO_4 \cdot 7H_2O$	"	9.3	8.4
Zn	$ZnSO_4 \cdot 7H_2O$	"	8.6	9.0

Table 2. Effect of metals on yields of toxin A in *P. aeruginosa* strain PA-103

	% Toxin A yields[a]			
	1 µg/ml		5 µg/ml	
Metal	Exp. 1	Exp. 2	Exp. 1	Exp. 2
Ba	105	97	125	119
Ca	112	122	107	102
Cu	113	108	112	117
Co	96	92	107	115
Fe	13	19	14	14
Mg	107	115	104	99
Zn	107	101	110	108

[a]Toxin A yields are based on the acid-insoluble radioactivity counts per min from the ADP-ribosyl transferase assay where counts per min from the culture containing no added metal was equivalent to 100%.

Appendix H. Detection of cross-reactivity between Pseudomonas aeruginosa toxin A, diphtheria toxin and its A fragment using a reversed passive hemagglutination assay.

Efforts have proven futile to show any immunological cross-reactivity between P. aeruginosa toxin A and diphtheria toxin or its A fragment. The methods employed have included Ouchterloney immunodiffusion analysis (4), enzyme neutralization (2), and the neutralization of tissue culture cytotoxicity (6).

Toxin A and diphtheria toxin differ from one another in a number of characteristics including cell specificity (6), amino acid compositions (1, 4), and mechanisms of potentiation (9). However, the ADP-ribosyl transferase activities of the two toxins are remarkably similar if not identical (1, 3). Fragment A from diphtheria toxin and a 26,000-27,000 dalton enzymatically active fragment of toxin A have similar Michaelis constants (Km's) for NAD and EF-2 (8 μM and 0.15 μM, respectively) and similar inhibition constants (Ki's) for a variety of analogs of NAD (1). In addition, the ADP-ribosyl transferase reaction catalyzed by fragment A can be reversed by toxin A or its enzymatically active fragment or vice versa (1, 3). Although the active fragments from the two toxins have different amino acid compositions both quantitatively and qualitatively (1), it would not be surprising if the catalytic centers of the fragments are similar or identical. If this is the case, very sensitive assay systems may be needed to detect any immunological cross-reactivity between the two toxins (or their active fragments). In this investigation, an attempt was made

to detect reactivity of A antitoxin with diphtheria toxin and its A fragment using a RPHA assay (Appendix B).

Diphtheria toxin was prepared in Dr. B. Iglewski's laboratory as previously described (5). Fragment A was obtained by limited proteolysis of diphtheria toxin using trypsin followed by reduction (5). Serial two-fold dilutions of diphtheria toxin and fragment A were made in 0.9% saline containing BSA (0.1 mg/ml).

The RPHA assay is reproducibly capable of detecting quantities of toxin A as small as 0.1 ng (Table 1). When diphtheria toxin was tested in the assay, agglutination of the antitoxin A sensitized SRBC was apparent at dilutions containing up to 25 ng of diphtheria toxin. Fragment A also caused agglutination of the sensitized SRBC at concentrations containing up to 10 ng (Table 1). Thus this assay system, using purified A antitoxin immunoglobulin linked to formalinized SRBC, was able to detect reactivity between P. aeruginosa toxin A and diphtheria toxin and its A fragment.

Fragment A, which is about one-third as large as diphtheria toxin (23,000 versus 63,000 daltons), should contain about three times as much of the catalytic center per unit weight as diphtheria toxin. Thus, the fragment A should react in the RPHA at lower concentrations than the whole toxin. The results obtained were consistent with this calculation (Table 1). Fragment A caused hemagglutination at concentrations as small as 10 ng while it took 2.5 times more diphtheria toxin (25 ng) before hemagglutination was observed. P. aeruginosa toxin A caused agglutination of the sensitized SRBC at concentrations 1/100 and 1/250 as small as the concentrations

of fragment A and diphtheria toxin required to cause hemagglutination.

These results provide evidence for the immunological cross-reactivity between toxin A of P. aeruginosa and diphtheria toxin and its A fragment. The reciprocal experiment using antitoxin (diphtheria toxin or toxoid) linked to SRBC to detect cross-reactivity with P. aeruginosa toxin A or its active fragment has not been performed. Two major problems complicate the completion of this latter study. First, diphtheria toxin is so toxic to most animals that immunogenic doses are lethal (7). One can immunize mice (8) or rats but the large numbers of animals required to obtain sufficient antiserum would make this study unfeasible for most laboratories. The second problem that complicates the study of determining immunological cross-reactivity between diphtheria toxin (and its A fragment) and toxin A is that animals immunized with diphtheria toxoid have very low titers to fragment A (7). Presumably, the A fragment is buried within the toxoid molecule (7).

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Table 1. Immunological cross-reactivity between P. aeruginosa toxin A and diphtheria toxin and its A fragment using a reversed passive hemagglutination assay

Preparation	RPHA endpoint[a]
toxin A	0.1 ng
diphtheria toxin	25 ng
fragment A of diphtheria toxin	10 ng

[a]The RPHA endpoint represented the smallest quantity of the material tested that caused complete agglutination of the sensitized SRBC.

Appendix I. Characterization of a Pseudomonas aeruginosa hemagglutinin.

The presence of a newly discovered P. aeruginosa extracellular factor with hemagglutinating properties was reported in paper 3 of this thesis. A few of the properties of the HA were investigated in the present study including heat stability, stability to dialysis and protease susceptibility.

P. aeruginosa strain WR-9 was grown in TSBD medium for 22 h as previously described (paper 3). An aliquot of the supernatant containing the HA was dialysed overnight against 0.01M Tris·HCl, pH 8.0 at 4° C. The HA titer of the supernatant was not changed after this dialysis (Table 1). Thus the HA was non-dialysable which indicates that the HA as it was found in crude supernatant fluid was probably greater than about 10,000 daltons in molecular weight and its activity (ability to agglutinate SRBC) was stable to this treatment. An alternative explanation is that the HA may be complexed with larger molecules.

The HA was also tested for its heat stability. An aliquot of the supernatant was kept on ice while another aliquot was heated to 100° C for 15 min. The heat treated HA was then cooled in an ice bath. The HA titers of the heat treated and the non-treated samples were identical (Table 1) indicating HA activity is relatively heat stable.

Finally, the proteases trypsin and pronase were tested for their effect on the HA activity. Stock solutions (1 mg/ml) of trypsin and

pronase (both from Sigma Chemical Corp.) in 0.05 M Tris·HCl, pH 8.0, were prepared. Two µg of each protease preparation were added to 100 µl of crude supernatant from strain WR-9 (the supernatant contained 150 µg protein/ml). Controls consisted of crude supernatant fluid without the proteases. The supernatants (with and without the proteases) were incubated for 30 min at 37° C. Following this incubation, the supernatants were chilled in an ice bath, then serial dilutions were made and tested for HA activity as previously described (paper 3). The HA activity was resistant to the actions of trypsin and pronase (Table 1). Similar results were obtained when the final concentrations of trypsin and pronase were increased to 4 µg per 100 µl supernatant. Neither pronase or trypsin had any detectable effects on the integrity of the formalinized SRBC.

In conclusion, the HA as it existed in a crude supernatant of P. aeruginosa strain WR-9 was non-dialysable, heat stable and protease resistant. It must be emphasized that these experiments were performed using a crude preparation of the HA. The HA may behave differently with regards to the parameters studied in this report when it is purified away from contaminating substances present in the culture supernatant.

Table 1. Stability to dialysis, heat stability, and protease susceptibility of the hemagglutinin from P. aeruginosa strain WR-9

		HA titer	
A.	Stability to dialysis	Before dialysis	After dialysis
		512	512
B.	Heat stability	Before heating	After heating
		256	256
C.	Protease susceptibility		
		Before trypsin	After trypsin
		256	256
		Before pronase	After pronase
		256	256

Appendix J. Pseudomonas aeruginosa strains that produce both toxin A and exoenzyme S.

Toxin A and exoenzyme S are P. aeruginosa ADP-ribosyl transferases that differ in a number of properties (paper 4). In all probability, these enzymes are coded for by separate genes. It is possible that individual strains of P. aeruginosa will produce both toxin A and exoenzyme S. This report investigates such a possibility.

Five strains of P. aeruginosa were used in this study. Strains PA-103 and 388 were used as A and S producing controls, respectively. The other 3 strains (PA-101, Ps-92 and PA-108) were the kind gift of Dr. J. Sadoff, Walter Reed Army Institute of Research.

The results of the Elek test (paper 1) showed that PA-101 and Ps-92 as well as the control strain, PA-103, were positive and thus produced toxin A (Table 1). Strain PA-108 was negative in this assay as was the negative control strain 388. A negative result in the Elek test does not necessarily mean that a strain is toxin A^- (papers 1 and Appendix B).

It is now well established that toxin A exists as a toxic proenzyme that must be potentiated before full expression of its enzymatic activity is obtained (1, 2, 3). The method used in this study to potentiate the enzymatic activity present in crude supernatants was to treat the supernatants with 4 M urea and 1% DTT (paper 2). Although such treatment potentiates the enzymatic activity of toxin A it partially destroys the enzymatic activity of crude exoenzyme S

(paper 4).

When the P. aeruginosa strains were grown in liquid TSBD medium without NTA, the ADP-ribosyl transferase activity in 22 h cultural supernatants was greater in all 5 strains upon potentiation with urea and DTT (Table 2). Strains PA-103 and Ps-92 showed the greatest potentiation, while the ADP-ribosyl transferase activity from strain PA-101 was increased about 5-fold. The enzymatic activity of strain PA-108 was only slightly increased following potentiation (Table 2). Surprisingly, the ADP-ribosyl transferase activity from strain 388 (grown in the absence of NTA) was increased by urea and DTT treatment. Therefore, on the basis of this experiment, it appears as though strains PA-103 (positive control), PA-92, PA-108, PA-101 and 388 all produced some toxin A.

Neutralization experiments with anti-A were performed to determine if the ADP-ribosyl transferase activity in the supernatants from the strains grown in TSBD (without NTA) medium were indeed due to toxin A. The ADP-ribosyl transferase activity in the supernatants following pretreatment with urea and DTT, from strains PA-103, PA-101, Ps-92 and PA-108 were neutralized by anti-A antiserum (Table 2). The neutralization was at least 95% complete in these supernatants. Although the CPM were low, the ADP-ribosyl transferase activity from strain 388 grown in the absence of NTA was also neutralized by anti-A antiserum (Table 2). Thus, on the basis of the Elek test, the potentiation experiment, and enzyme neutralization by A antitoxin strains PA-103, PA-101, PA-108, Ps92 and 388 produced toxin A when grown in the absence of NTA.

All strains, except PA-103, produced exoenzyme S (Table 4). In the presence of TSBD +10 mM NTA strains 388, PA-101, PA-108, and Ps-92 produced far more ADP-ribosyl transferase activity than could be explained by the formation of ADPR-EF-2 (paper 4). In addition, the enzymatic activity in the supernatants grown in the presence of NTA was decreased rather than potentiated by urea + DTT treatment (Table 4). Furthermore, the ADP-ribosyl transferase activity was far greater in supernatants from strains grown in the presence of NTA than in its absence (Tables 2 and 4), a characteristic that has been extensively studied in strain 388, a known exoenzyme S producer (paper 6). Strain PA-103 reacted similarly in TSBD medium and in TSBD + NTA medium (Tables 2 and 4). Thus far it appears that strain PA-103 produces toxin A but not exoenzyme S.

The evidence in this report shows that some strains of P. aeruginosa have the ability to produce both toxin A and exoenzyme S. The most surprising result of this investigation was the finding that strain 388, the prototype S producer used in Dr. Iglewski's laboratory, produced small amounts of toxin A. Extrapolation to previous data (paper 3) indicates that the levels of toxin A produced by strain 388 in vitro are about 0.3 µg/ml which represents about 1% of the toxin A levels produced by strain PA-103. Toxin A was not detected in skin extracts and sera from experimental animals infected with strain 388 (paper 5). Perhaps the use of more sensitive techniques would have detected the presence of toxin A in these animals.

References

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Table 1. Detection of toxin A producing P. aeruginosa strains by the Elek test

Strain	Elek test
388 (S control)	-
PA-103 (A control)	+
PA-101	+
Ps-92	+
PA-108	-

Table 2. ADP-ribosyl transferase activity in supernatants of P. aeruginosa strains grown in TSBD medium without NTA

	ADP-ribosyl transferase activity (CPM)	
Strain	$+H_2O$	+(urea + DTT)
388	0	150
PA-103	20	5918
PA-101	212	975
Ps-92	0	1183
PA-108	365	495

Table 3. Neutralization of ADP-ribosyl transferase activity in supernatants from P. aeruginosa cultures grown in the absence of NTA by A antitoxin

	ADP-ribosyl transferase activity (CPM)[a]		
Strain	+Normal rabbit serum	+A antitoxin	
388	66	2	(97)[c]
PA-103[b]	704	20	(97)
PA-101	560	0	(100)
Ps-92	415	18	(95)
PA-108	684	0	(100)

[a]The neutralization of the ADP-ribosyl transferase activity was performed as described in paper 6.

[b]The crude supernatant of strain PA-103 was diluted 1:5 in PBS so that the CPM in the presence of NRS fell within the linear range of the ADP-ribosyl transferase assay (paper 6).

[c]The numbers in parenthesis represent the % decrease in ADP-ribosyl transferase activity when the supernatants were treated with A antitoxin as compared to normal rabbit serum.

Table 4. ADP-ribosyl transferase activity in supernatants of *P. aeruginosa* strains grown in TSBD medium containing 10 mM NTA

Strain	ADP-ribosyl transferase activity (CPM)	
	$+H_2O$	+(urea + DTT)
388	36204	16850
PA-103	481	5214
PA-101	80425	28450
Ps-92	10818	7004
PA-108	39342	10115

VI. Abbreviations

A	toxin A
ADP	adenosine diphosphate
ADPR	adenosine diphosphate-ribose
AMP	adenosine monophosphate
BDB	Bis-diazotized benzidine
BSA	bovine serum albumin
CF	cystic fibrosis
CFU	colony forming unit
Ci	curie (2.22 disintegrations per second)
CPM	counts per minute
DE	diethylaminoethyl
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EF	elongation factor
GARG	goat anti-rabbit gamma globulin
HA	hemagglutinin
LD_{50}	mean lethal dose
LPS	lipopolysaccharide
MSG	monosodium glutamate
NAC	N-acetyl cysteine
NAD	nicotinamide adenine dinucleotide
NMN	nicotinamide mononucleotide
NRS	normal rabbit serum

NSS	normal sheep serum
OD	optical density
P.	Pseudomonas
PGT	pantothenate, glutamic acid and tryptophan medium
PP	proteose peptone
PPD	proteose peptone dialysate
RPHA	reversed passive hemagglutination
S	exoenzyme S
SDS	sodium dodecyl sulfate
SRBC	sheep red blood cells
TCA	trichloroacetic acid
TSB	trypticase soy broth
TSBD	trypticase soy broth dialysate

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