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Growth of *Pseudomonas aeruginosa* in Normal and Burned Skin Extract: Role of Extracellular Proteases

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Growth curves and mean generation times (MGT) were determined for *Pseudomonas aeruginosa* strain M-2 (protease +) and strain PA-103 (protease ±) in burned skin extract (BSE) and in normal skin extract (NSE). Strain M-2 grew on NSE or BSE with an MGT of 30 min. Strain PA-103 grew in NSE at a similar MGT; however, PA-103 in BSE had a MGT of 65 min. When protease was added to BSE, PA-103 grew as rapidly as M-2. When ammonium sulfate was added to inhibit protease production, the MGT of M-2 slowed to that of PA-103 in BSE. The MGT of PA-103 in amino acid-supplemented BSE was similar to that of M-2 in both BSE and NSE. These data suggest that protease may serve as a virulence factor by modifying the available nutrients in burned skin. As a result, nutrients are formed that permit an enhanced growth rate and a more rapid establishment of the infection in the host.

Burned skin wounds seem to be especially susceptible to infection by *Pseudomonas aeruginosa* (8). The pathogenesis of *P. aeruginosa* infections has been studied in experimentally produced burns in a burned mouse model (14). From these studies it was demonstrated that the burned skin site allowed for the initial colonization and proliferation of the organism in vivo. Subcutaneous inoculation at the burned site of as few as 10 *P. aeruginosa* cells was 100% lethal to burned mice. However, for other organisms, such as *Escherichia coli, Klebsiella* spp., *Staphylococcus aureus*, and *Candida albicans*, a similar degree of lethality occurred only after subcutaneous inoculation with $>9 \times 10^7$ organisms. In normal mouse skin, subcutaneous inoculation of any of these organisms, including *Pseudomonas*, showed the lower degree of susceptibility. Therefore, it seemed that skin that was burned was particularly modified for the growth of *P. aeruginosa*.

Why *P. aeruginosa* is more virulent in a burned wound than other microorganisms is not completely known. It has been reported that unique products of *P. aeruginosa* such as exotoxin A (9, 12) and proteases (2, 10, 12) may contribute to its pathogenesis in burned skin. Two strains of *P. aeruginosa* being used in our laboratory have shown different degrees of infectivity in our experimental mouse model. One strain, M-2, a toxin and protease producer, has been shown to have a 50% lethal dose (LD_{50}) of <10 organisms when subcutaneously inoculated into mice at the burned site (14). Another strain, PA-103, a toxin producer but a poor protease producer, showed an LD_{50} of $>10^6$ cells in the same animal model (unpublished data). When protease was added, PA-103 showed a significant reduction in LD_{50}. Thus, it was suggested that proteases might be virulence factors (2, 10).

Proteases, as extracellular enzymes of *P. aeruginosa*, provide essential nutrients for its growth. Specifically these enzymes convert large molecules of protein that cannot penetrate the cell envelope into smaller molecules of peptides that can be transported into the cell. Proteases may serve to provide nitrogen, carbon, and energy to *Pseudomonas* in cases where proteins are the sole source of one or more of these requirements. Alternative nitrogen sources, such as ammonium sulfate, have been shown to decrease protease production by this organism (5, 6).

Since proteases have been suggested as possible virulence factors for *P. aeruginosa* in the experimental burned mouse model, we chose to investigate whether their effect was to provide nutrients and support the growth of this organism in burned skin. To measure the effect of proteases on the growth dynamics of *Pseudomonas* as a separate phenomenon, we eliminated other competing factors by measuring growth in vitro in extracts of burned or normal skin.

MATERIALS AND METHODS

Organisms. *P. aeruginosa* strain M-2, which produces exotoxin A and extracellular proteases, was originally isolated from the intestinal tract of normal CF-

1 mice (14). *P. aeruginosa* strain PA-103, which produces exotoxin A but is deficient in protease production was originally obtained from P. V. Liu, University of Louisville (5). Although these strains are not isogenic, they have been characterized together in the burned mouse model and they show a relationship between elaboration of exoproducts and virulence (2, 12). Both strains were maintained on brain heart infusion slants. For preparation of inoculum, each organism was grown overnight in a basal salts medium (1), harvested, washed one time in 0.005 M phosphate buffer (pH 7.0), and suspended in the same buffer.

Protease determination. The quantities of extracellular proteases produced by each strain of *Pseudomonas* was compared by using Trypticase soy agar plates containing 3% skim milk as the protein substrate (5). Strain M-2 showed a wide diameter zone of clearing around each colony at 24 h. Strain PA-103 showed no clearing at 24 h. At 48 h, a small zone of clearing became detectable around each colony.

The quantity of proteases present in the growth media (see below) at 26 h was assayed spectrophotometrically by using hide powder blue as substrate. Skin extracts were removed from the growth flasks and filter sterilized, and proteolytic activity was measured according to published procedures (11). Hide powder blue was obtained from Sigma, St. Louis, Mo.

NSE and BSE. Normal skin extract (NSE) was prepared by shaving the back of a mouse and cleansing the area with ethanol. After sacrificing the animal by cervical dislocation, the shaven skin was aseptically removed, homogenized in 10 ml of saline for 5 min, and centrifuged for 1 h at 12,000 rpm. The top layer containing lipids was removed by aspiration. The clear middle layer of NSE was then decanted, filter sterilized, and used in these experiments. As described previously, the burn wound was inflicted by adding ethanol to the shaven skin area and igniting for 10 s (3). The animal was sacrificed, and the skin in the burned area was used to prepare burned skin extract (BSE) by the same method used to make NSE.

Growth rate determinations. A 13-ml portion of sterile NSE or sterile BSE containing a protein concentration of 100 μg/ml, was added to a 500-ml sidearm flask. Results were found to be the same by using either undiluted extracts or extracts diluted with sterile distilled water to 100 μg of protein per ml. A suspension of washed cells (0.1 ml) was added to give a final cell concentration of approximately 5×10^2 cells per ml in the extract. The pH of the dilute NSE or BSE was approximately 7.0 and did not have to be adjusted to neutral. The flask was incubated at 37°C in a shaking water bath. At various intervals, usually 1 h, a sample was removed for quantitative plate count, and the pH was determined. If during logarithmic growth the pH was found to vary from neutral by greater than 0.5, either NaOH or HCl was added to bring the pH to 7.0. The generation time or the rate of cell doubling was determined by viable plate counts of portions of skin extracts removed from the flask each hour for at least 12 h after inoculation. At least two generation times, each determined by repeating the experiment on different days, were used to calculate the mean generation time (MGT). Growth rates of *P. aeruginosa* are expressed as MGT.

Supplementation of BSE. In certain growth experiments, either protease, ammonium sulfate, or amino acids were added to the BSE growth media. Additions to the skin extract in the growth flask were made immediately before inoculation. The final concentration of crystalline protease (see below) was 100 μg/ml. Ammonium sulfate was added to a concentration of 5%. A basal medium Eagle amino acid mixture (100×) without glutamine, which was purchased from Flow Laboratories, Inc., was supplemented at 1× strength in the BSE growth media.

Protease. Crystalline *Pseudomonas* protease prepared from culture filtrates of *P. aeruginosa* IF03080 was a gift of K. Morihara, Osaka, Japan. The protease has a molecular weight of 48,000, migrated as a single band in electrophoresis, and showed proteolytic but not elastolytic activity. In growth studies purified protease was weighed and added to the skin extract in the growth flask. This protease has been designated alkaline protease or protease III by other investigators.

RESULTS

Comparisons of protease production by strains M-2 and PA-103 in BSE. Strains M-2 and PA-103, which both produce exotoxin A, differ in the amounts of extracellular proteases they produce. As shown in Table 1, PA-103 produced no detectable proteases in 26-h growth menstrum, whereas M-2 produced 4,000 U/ml per h by the hide powder blue assay. On skim-milk plates, a small clear zone was detected around PA-103 colonies after 48 h, suggesting that this strain may produce a small amount of proteases not measurable with the hide powder blue assay.

Growth of *P. aeruginosa* M-2 in NSE and BSE. When *P. aeruginosa* M-2 was inoculated into BSE or NSE, growth curves were generated (Fig. 1). In each extract, after a 2-h lag, the bacteria showed a logarithmic (log) growth phase which continued to a concentration of 1×10^8 cells per ml, at about 11 h after inoculation. During log growth, strain M-2 maintained a rapid growth rate with a MGT of 28 min in NSE and 30 min in BSE. In the latter medium, stationary growth was reached at 6.3×10^8 cells per ml, at 15 h, after which time the cell number remained constant. The bacteria did not show as sharp a tapering off of growth in NSE as in

TABLE 1. *Extracellular protease activity of P. aeruginosa strains M-2 and PA-103*

Strain	Activity in BSE after 26 h of growth[a]	Lytic zone around colony on skim milk agar plate after 48 h
M-2	4,000 U/ml per h	++++
PA-103	Not detectable	+

[a] Determined by hide powder blue assay.

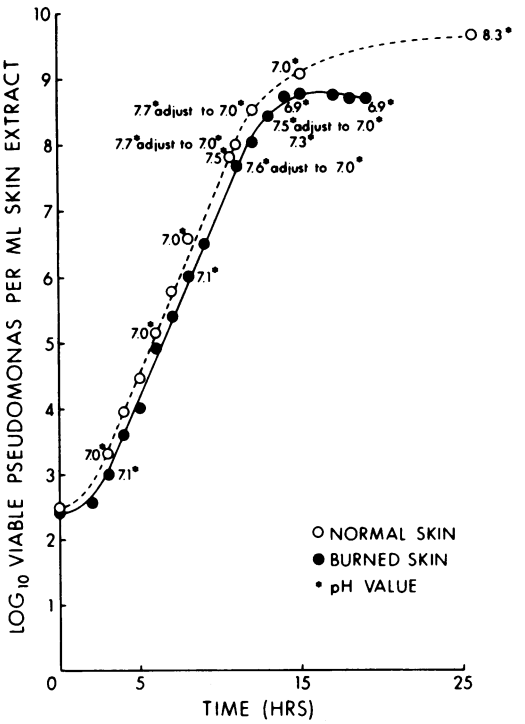

FIG. 1. *Growth of P. aeruginosa M-2, protease producer, in NSE and BSE. The organisms were grown at 37°C in a shaking water bath in 20 ml of skin extract in a 500-ml flask. The pH values were adjusted where indicated with HCl.* ○, *Growth in NSE;* ●, *growth in BSE.*

BSE. In NSE, a concentration of 1.2×10^9 cells per ml was present at 15 h, and this gradually continued to increase slightly to 4.5×10^9 cells per ml by 26 h. However, overall both media supported a similar maximum cell yield at stationary phase (within 13% of each other), and this yield required about 15 h of growth in both cases.

The unadjusted pH of BSE was approximately 7. As seen in Fig. 1, bacterial growth in these extracts did not change the initial pH to a concentration of 3.7×10^6 cells per ml. When the cell concentration increased above this number, the pH became alkaline. As the pH increased to 7.5 or greater, HCl was added to the medium to adjust the pH to neutral. Between a concentration of 3.7×10^6 and 5.5×10^8 cells per ml, the pH of both media continued to rise, and several adjustments were made. When log growth tapered off, the pH remained as adjusted. After several hours in stationary phase the pH usually became 8.3. This is shown in Fig. 1 for NSE at 25 h and was observed in repeat growth curve experiments to occur both in NSE and BSE after 25 h or more. The increase in pH was assumed to reflect accumulation of alkaline products of bacterial growth which overcame any natural buffer capacity of the skin extract.

Growth of *P. aeruginosa* PA-103 in NSE and BSE. When a different strain of *P. aeruginosa*, PA-103, which produces only low amounts of extracellular proteases, was inoculated at a concentration of 2.8×10^2 cells per ml into NSE and BSE, the growth curves shown in Fig. 2 were observed. A short period of lag, less than 2 h in NSE and none in BSE, was followed by log growth which was different in NSE and BSE. In normal extract, log growth was characterized by an MGT of 29 min, similar to those observed for M-2, and continued until a cell concentration of 1.0×10^9 cells per ml was reached. On the other hand, PA-103 growing in BSE showed biphasic log growth. In phase A, between 0 and 12 h until a cell concentration of 5×10^5 cells per ml was reached, the MGT was 65 min. In phase B at between 12 and 17 h and at concentrations between 5×10^5 and 1.5×10^8 cells per ml, the growth rate increased to a 30-min doubling time. Phase B was characterized by an increase in pH above the constant pH 7.0 of phase A. From the

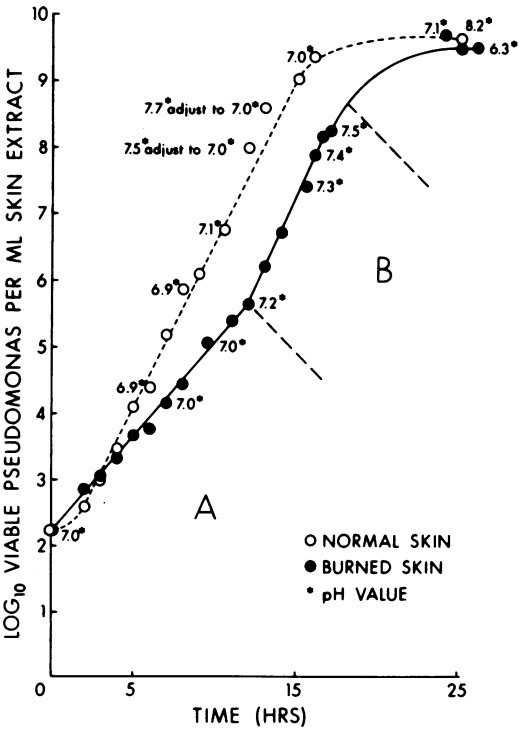

FIG. 2. *Growth of P. aeruginosa PA-103, a low protease producer, in NSE and BSE. Organisms were grown in 20 ml of skin extract in a 500-ml flask at 37°C in a shaking water bath. The pH values were adjusted where indicated with HCl.* ○, *Growth in NSE;* ●, *growth in BSE.*

first point taken in phase B (pH 7.2), until the last (pH 7.5), the pH remained alkaline. The alkalinity of log phase B contrasted with the return to neutral pH observed in stationary phase, followed by a drop to pH 6.3 at 26 h. Cells growing in NSE also produced an alkaline pH during the latter part of log growth at concentrations between 3×10^6 and 1×10^9 cells per ml. In NSE, the pH became alkaline, usually reaching pH 8.2 at 26 h. Cells growing in NSE reached stationary growth in 15 h with a maximum yield of about 1×10^9 cells per ml. On the other hand, cells in BSE did not reach stationary growth until 22 h, at which time a similar maximum yield of 1×10^9 cells per ml was achieved.

Growth of strain M-2 in BSE plus ammonium sulfate. Since strain M-2 produces higher amounts of extracellular protease than PA-103, it was thought that these proteases were responsible for the faster growth rate of strain M-2 on BSE. To test this hypothesis, 5% ammonium sulfate was added to BSE to inhibit the production of extracellular proteases. As shown in Fig. 3, strain M-2 showed quite different growth characteristics in BSE containing 5% ammonium sulfate. A 5-hour lag period was followed by log growth which continued through hour 25, at which point the bacterial concentration was 2.1×10^7 cells per ml. Log growth of M-2 in the presence of ammonium sulfate was considerably slower, with an MGT of 74 min, than the MGT of 30 min observed (Fig. 1) for M-2 in BSE without ammonium sulfate, but the slower MGT was similar to the MGT of 65 min for PA-103 (Fig. 2) in BSE alone. Measurements of the pH of the medium taken during growth showed that in contrast to growth in BSE, growth on BSE plus ammonium sulfate failed to produce an alkaline shift in the medium (Fig. 3). Indeed, the BSE plus ammonium sulfate tended to become slightly acidic, and it was necessary to add NaOH from time to time to neutralize the extract. This is in contrast to the growth curves in Fig. 1 and 2, which became alkaline in late log phase and suggested that different products of metabolism were being produced.

Growth of strain PA-103 in BSE plus purified protease. PA-103 may have grown slowly in BSE because it produced only a low amount of protease which could not provide sufficient nutrition from available protein. If this were the case, the addition of purified protease might correct this deficiency. Figure 4 shows this was the case. When purified *Pseudomonas* protease was added to BSE, PA-103 showed a 2-h lag and then entered the log growth phase until a concentration of 2×10^8 cells/ml was reached. The

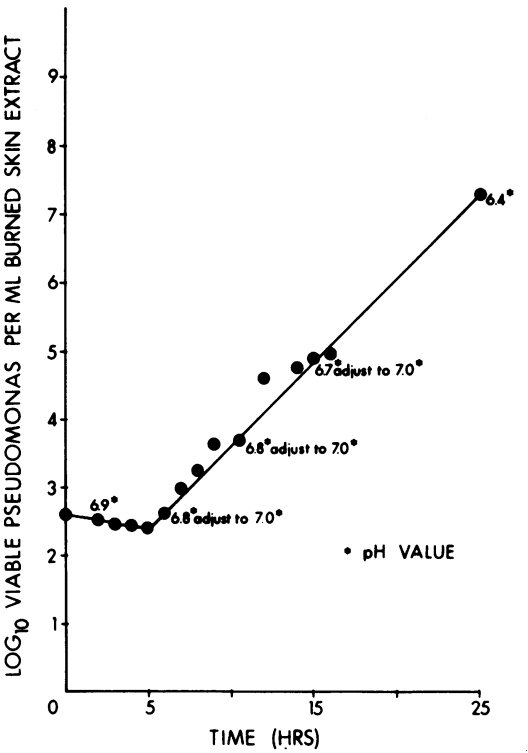

FIG. 3. *Growth of P. aeruginosa M-2 in BSE-supplemented ammonium sulfate. Organisms were grown in 20 ml of skin extract plus 5% ammonium sulfate in a 500-ml flask at 37°C in a shaking water bath. The pH values were adjusted where indicated with NaOH.*

MGT of 29 min was comparable to that of M-2. In this supplemented medium, PA-103 reached stationary growth in 15 h at a concentration of 2×10^9 cells per ml. The pH of the extract was observed to become alkaline during the late part of the log phase. This was followed by a shift to an acid pH in early stationary phase, which was replaced again with an alkaline pH at 27 h. A similar growth curve was observed for PA-103 in BSE supplemented with thermolysin, an extracellular protease of *Bacillus* (data not shown).

Growth of strain PA-103 in BSE plus amino acids. Since the increase in the MGT of PA-103 in protease-supplemented BSE might possibly have been due to destruction of an inhibitor of growth rather than to production of nutrients, a similar growth study was made in BSE supplemented with an amino acid mixture (1× basal medium Eagle without glutamine). The results in Fig. 5 show an increased MGT to 32 min. The pH remained relatively stable throughout the growth curve, suggesting an equal production of acid and alkaline products.

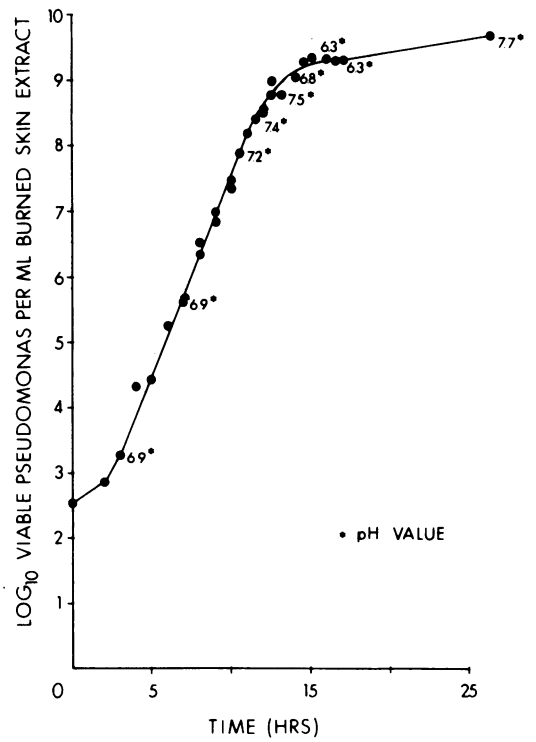

FIG. 4. *Growth of P. aeruginosa PA-103 in BSE plus purified protease. Organisms were grown in 20 ml of skin extract supplemented with purified Pseudomonas protease which was present at a concentration of 100 μg of protein per ml in a 500-ml flask at 38°C in a shaking water bath.*

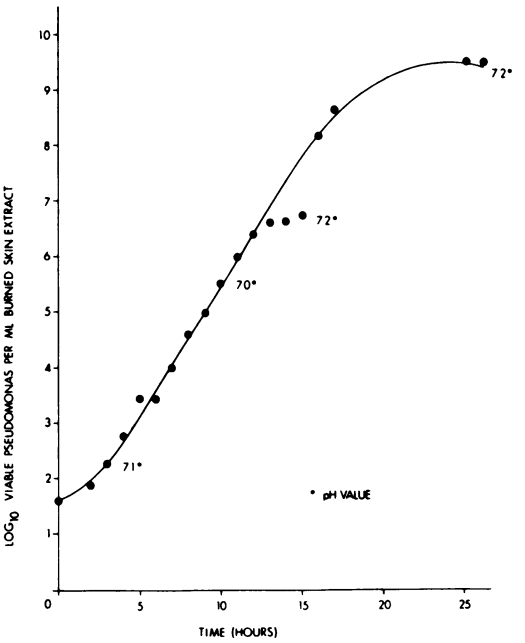

FIG. 5. *Growth in P. aeruginosa PA-103 in BSE plus amino acids. Organisms were grown in 20 ml of BSE supplemented with basal medium Eagle amino acid mixture without glutamine, which was present at a 1× concentration.*

DISCUSSION

Several recent reports have demonstrated that proteases of *P. aeruginosa* play a role in the virulence of this microorganism and are part of the pathogenic process in these infections. By using a burned mouse model, Snell et al. (12) showed that although infections with low inocula of *P. aeruginosa* PA-103 caused no reduction of EF-2 levels in the livers of infected mice, significant reductions were observed when these inocula were supplemented with injections of small amounts of protease. Other studies using the same model have shown significant enhancement of mortality when strain PA-103 challenge was supplemented with protease or elastase compared with non-enzyme-supplemented challenge (2).

In the same study, neutralization of protease activity by local injection of the protease-inhibiting serum protein, α-2-macroglobulin, enhanced the survival of the mice infected with a protease-producing strain. Other investigators have demonstrated decreased LD_{50} values when burned mice were challenged with protease-deficient mutants of a protease-producing wild-type parent (10).

The studies cited above have established the role of protease as a virulence-associated factor in *P. aeruginosa* infections. The mechanism by which proteases act as virulence factors remains unclear. We know, however, that proteases supply *P. aeruginosa* with small peptides of usable size which can be transported into the cell (13). The lack of such activity in the presence of proteins and large peptides that are the primary nitrogen or carbon source could starve the cells or force utilization of poorer sources of nutrients, which would lead to a decreased growth rate. Such a critical role for extracellular proteases was supported by the experiments reported here.

No difference in MGT was observed when the protease-producing strain M-2 was grown in either NSE or BSE (Fig. 1). However, a difference was apparent when the protease-deficient strain PA-103 was grown on these media (Fig. 2). Whereas the MGT for both strains (28 min for M-2, 29 min for PA-103) was similar when grown on NSE, PA-103 showed a biphasic growth curve when grown in BSE. In contrast to the 30-min MGT observed throughout the

whole log phase when M-2 was grown in BSE, PA-103 required 65 min in phase A growth in BSE. This suggested that for the protease-deficient strain the BSE might be nutritionally deficient. Supplementation of the BSE with protease increased the MGT of PA-103 to 29 min, which was comparable to that of M-2 in BSE. Conversely, inhibition of the proteolytic activity produced by M-2 growing in BSE plus 5% ammonium sulfate increased the MGT of M-2 to 74 min, similar to that of protease-deficient PA-103 (65 min) in log phase A. Ammonium sulfate added to M-2 growing in NSE or to either M-2 or PA-103 growing in nutrient broth had no effect on the growth rate (data not shown), suggesting that the effects of ammonium ions were specifically directed to protease inhibition, as has been previously reported (6).

These data suggested that the protease may act by producing more utilizable nutritional substrates for a pseudomonad growing in a nutritionally deficient medium, such as BSE, thereby enhancing growth. As shown in Fig. 5, supplementation of BSE with amino acids increased the MGT of PA-103 to close to that of M-2 grown in BSE or PA-103 grown in BSE supplemented with protease. Therefore, the presence of sufficient protease concentrations in the menstrum was not necessary to achieve an increased MGT for PA-103 in BSE. This suggests that growth stimulation of PA-103 by supplemented protease was not the result of inactivation of a protein growth inhibitor, but rather production of protein end products such as small peptides and amino acids, which could be utilized as preferred nutrients by the pseudomonad to satisfy its nutritional requirements.

The changes in pH observed during the growth curves are important and require comment. We believe the pH shifts to alkalinity during the growth curves indicated the presence of extracellular protease activities. Peptides produced by proteases in turn would be metabolized by the cells for energy, carbon, and nitrogen, with accumulation of the end product, ammonia. Accumulation of ammonia would result in the alkaline shift observed to occur in vivo in human burns infected with *P. aeruginosa* (4). In the present studies the alkaline shift seemed to be a sensitive indicator of the presence of proteolytic activity. In Fig. 1 and 4, an alkaline shift occurred above a concentration of 5×10^6 cells per ml for the three growth curves in which protease was present, either produced by M-2 or added to the medium for PA-103. Although at this time protease could not be detected in the growth medium with the hide blue assay because of limited sensitivity, we believed that the presence of protease was detectable indirectly by the otherwise unexplainable shift to alkalinity which occurred repeatedly at the critical concentration of 5×10^6 cells per ml. It is possible that protease activity and formation of ammonium ions were occurring before the critical concentration of 5×10^6 cells per ml, but that the innate buffering capacity of the skin extract maintained a neutral pH in the medium. Once a concentration of 5×10^6 cells per ml was reached, enough ammonium had been produced to overcome the buffering capacity of the extract. In Fig. 2, the alkaline shift occurred at the same critical concentration of 5×10^6 cells per ml. Interestingly, this shift coincided with the increased growth rate of PA-103 in BSE. This was reproducible in six separate experiments. PA-103 produced only low amounts of protease (Table 1). One explanation for the biphasic growth shift of PA-103 is that enough protease had accumulated at a cell concentration of 5×10^6 cells per ml to produce a quantity of peptides with sufficient nutrient value that simultaneously supported an increased growth rate and caused an alkaline shift. In contrast, the growth curve of M-2 in BSE plus ammonium sulfate, which inhibited protease production, showed no alkaline shift. Without extracellular proteases, perhaps *Pseudomonas* utilized other types of nutrients in BSE that were metabolized by pathways which resulted in acid products and a slower growth rate.

It was interesting to observe that NSE allowed a very rapid growth rate for both strains of *P. aeruginosa*. Yet as an intact structure, normal skin is a most effective barrier to colonization and multiplication of *P. aeruginosa*. On the other hand, BSE was altered from NSE. Possibly because of a change in available nutrients it no longer supported rapid growth of the low protease-producing strain.

For a strain of *P. aeruginosa* that produced normal quantities of proteases, its increased growth rate on a burned skin site could favor its successful establishment in a host. In these studies, an initial inoculum of approximately 300 cells per ml of M-2 in BSE reached a concentration of 10^6 cells per ml in under 7 h. On the other hand, PA-103 in identical conditions required over 14 h to reach 10^6 cells per ml. Moreover, these in vitro growth studies favored more rapid growth than occurs in vivo. A previous study measured the doubling time of M-2 in the burned skin of an infected mouse as 72 min (D. Stieritz, Ph.D. Thesis, University of Cincinnati, Cincinnati, Ohio). Although the in vivo generation time of PA-103 was not determined, it might be expected to be at least twice as long as strain M-2. Thus, for PA-103, depending on the inoc-

ulum size administered at the burned skin site, a concentration of bacteria of 10^6 cells on the skin site might well require over 30 h (assuming 200 initial cells with a 144-min generation time). This would allow considerable time for the host to mount a protective response. The higher LD_{50} of 10^6 cells per skin site for PA-103 (unpublished data) compared with 10 cells per skin site for M-2 (14) was consistent with an enhancement of virulence by faster growth rate.

Another study consistent with enhancement of virulence in protease-producing strains showed that in a burned mouse model fewer viable bacteria were found in the blood of animals infected with protease-deficient *P. aeruginosa* than were found in the blood of animals infected with protease-producing strains (10). In addition, antiprotease serum therapy enhanced survival of animals infected with protease-producing strains. The authors suggested that protease acted by contributing to the invasiveness of *P. aeruginosa*. This possibility had been previously speculated by others (2, 12) by using burned mouse models.

Data presented here are consistent with contributing a role to protease in enhancing the appearance of bacteria in the bloodstream from the skin site of inoculation. Another investigation has shown that *P. aeruginosa* are not readily transmitted to the blood until a wound level of greater than 10^5 organisms per g of wound tissue is reached (13). Preferential nutrients derived from proteolysis of burned skin in vivo might be expected, based on the in vitro data in BSE, to facilitate the growth of the bacteria to the critical mass of 10^5 cells per g of tissue required for systemic invasion. Thus, rapid growth at the skin site may be considered a mechanism by which the virulence of protease-producing *P. aeruginosa* is enhanced.

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