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Effects of α-Amylase on In Vitro Growth of *Legionella pneumophila*

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Sterile parotid saliva inhibited growth of *Legionella pneumophila* on solid media, and the salivary component involved in this inhibition has been shown to be amylase. Disk diffusion and well plate assays were used to study possible mechanisms for this effect. The amylolytic activity of saliva copurified with inhibitory activity, and both activities were sensitive to proteinase K digestion and heat treatment. In addition, purified α-amylase from several sources (bacteria, fungi, porcine pancreas, and human saliva) exhibited similar activity. Incorporation of charcoal or bovine serum albumin into media blocked inhibition by amylase. Replacement of Bacto-Agar with Noble agar (both from Difco Laboratories) prevented growth inhibition in the absence of starch. However, when corn starch was present with Noble agar, amylase-induced growth inhibition occurred. Purification of starch by washing with methanol eliminated some toxic component. The toxic component from starch could be recovered from the methanol wash and inhibited growth of *L. pneumophila* in the absence of amylase activity. The results suggest that toxic substances exist in media components which may be unmasked during salivary amylase digestion of starch. This effect may explain, in part, the difficulty in recovery of the organism from clinical specimens containing amylase.

The ubiquitous nature of *Legionella pneumophila* with pathogenic potential (4, 7, 22) would suggest that people are frequently exposed to this microorganism without overt infection. This, in conjunction with the increased risk of compromised patients (5, 18), would suggest the presence of innate host defense mechanisms in humans. The portal of entry for this organisms is generally accepted to be naso- or oropharynx, suggesting that secretions bathing these mucosal surfaces may provide the first line of defense against establishment of this pathogen. Preliminary studies in this laboratory (R. R. Arnold, R. D. Miller, R. F. Levine, and W. L. Dempsey, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B69, p. 26) have indicated that human parotid saliva contained a potent inhibitor of the growth of *L. pneumophila* that could not be attributed to the traditionally considered antimicrobial agents of exocrine fluids, including secretory immunoglobulin A, lactoferrin, lysozyme, lactoperoxidase, glycoprotein-aggregating factors, and certain complement components. Furthermore, the data from this study suggest that the component responsible for this inhibition is salivary amylase (C. A. Bortner, R. D. Miller, and R. R. Arnold, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, B151, p. 43). Amylase is found in high concentration in saliva and other mucosal secretions (15), and Clarke et al. (1) detected high levels of amylase in bronchial secretions of seriously ill patients.

Recent studies have suggested that amylase is capable of a potent and specific inhibition of *Neisseria gonorrhoeae*, apparently resulting in cell wall variants of the gonococcus (13, 14). *N. gonorrhoeae* and *L. pneumophila* appear to be uniquely sensitive to this amylase effect, as none of a broad range of other microbial strains tested were affected (13, unpublished observations). The present studies were designed to determine whether there is a direct effect on *L. pneumophila* as reported for *N. gonorrhoeae* or whether inhibition can be attributed to removal of the detoxifying effects of starch (17) on certain media. Such an effect of amylase could explain, in part, the difficulty in culturing from respiratory specimens (10, 20) and perhaps host resistance to legionellosis.

MATERIALS AND METHODS

Bacterial strains. *L. pneumophila* strains Bellingham-1, California-1, Knoxville-1, and Philadelphia-2 (all serogroup 1), Togus-1 (serogroup 2), Bloomington-2 (serogroup 3), Los Angeles-1 (serogroup 4), Dallas-1E (serogroup 5), and Chicago-2 (serogroup 6) were

obtained from R. Weaver, Centers for Disease Control, Atlanta, Ga. Cultures were maintained on GC-FC agar (21) plates at 37°C in 5% CO_2 and transferred weekly. Permanent stock cultures were stored at −70°C in tryptic soy broth (Difco Laboratories, Detroit, Mich.) containing 20% glycerol.

The Knoxville-1, Los Angeles-1, and Chicago-2 strains were also sequentially passaged through female Hartley guinea pigs to enhance virulence (designated Knoxville-1^v, Los Angeles-1^v, and Chicago-2^v, respectively). Approximately 10^8 bacteria were injected intraperitoneally, isolated from heart blood (or peritoneal fluid) 24 to 48 h later on buffered charcoal-yeast extract agar, and cultured for purity on buffered charcoal-yeast extract agar before subsequent passage. These strains were maintained as frozen stock cultures (as described above) and cultured on buffered charcoal-yeast extract or GC-FC agar when required.

Media. The primary medium used in this study was GC-FC agar (21), prepared from GC medium base (Difco) supplemented with ferric pyrophosphate (0.25 g/liter) and L-cysteine (0.4 g/liter). This medium contained the following (per liter): Proteose Peptone no. 3 (Difco), 15 g; NaCl, 5 g; K_2HPO_4, 4 g; KH_2PO_4, 1 g; corn starch, 1 g; and Bacto-Agar (Difco), 10 g. The buffered charcoal-yeast extract agar was prepared as previously described (2, 16). Modifications of these media will be described below.

Saliva and serum samples. Unstimulated human parotid saliva was collected from volunteers with an intraoral cup designed for that purpose (19). Guinea pig and mouse saliva were collected after pilocarpine stimulation (12). After centrifugation (10,000 × *g*, 10 min) to remove particulate material, samples were membrane filter sterilized (0.45-μm pore size). Saliva was generally tested on the day of collection, although storage at 4°C for over 1 year did not result in significant loss of activity. Serum was provided by human donors and was collected from mice and guinea pigs by cardiac puncture.

Chemicals. The following were obtained from Sigma Chemical Co., St. Louis, Mo.: α-amylase type I-A from porcine pancreas (specific activity, 1,000 U/mg of protein); α-amylase type II-A from *Bacillus subtilis* (specific activity, 1,900 U/mg of protein); α-amylase type IX-A from human saliva (specific activity, 860 U/mg of protein); and α-amylase type X-A from *Aspergillus oryzae* (specific activity, 144 U/mg of protein); trypsin type I from bovine pancreas (specific activity, 10,000 units per mg of protein); protease type VIII from *Bacillus subtilis* (subtilopeptidase A; specific activity, 9 U/mg of solid); and protease type V from *Streptomyces griseus* (pronase AS; specific activity, 1 U/mg of solid). Proteinase K from *Tritrachium album* (specific activity, 20 units per mg of protein) was obtained from EM Biochemicals, Darmstadt, West Germany. Neuraminidase from *Vibrio cholerae* (specific activity, 3.8 IU/mg of protein) was obtained from Calbiochem-Behring Corp., La Jolla, Calif.

Disk assay. Bacteria were washed from a GC-FC agar plate with sterile distilled water and grown in a 300-ml nephelometer flask containing 25 ml of GC-FC liquid medium. The broth was inoculated to give an initial turbidity of 35 Klett units with a Klett-Summerson colorimeter (660 filter) and incubated at 37°C on a New Brunswick Gyrotory shaker at 200 rpm. When the culture reached 75 Klett units, the cells were harvested by centrifugation (10,000 × *g*, 10 min) at room temperature, and the pellet was suspended in 5 ml of sterile distilled water. The cell suspension was spread evenly on GC-FC agar plates with cotton-tipped applicator sticks to achieve confluent growth. After 5 min to permit absorption of excess moisture, sterile analytical paper disks (6-mm diameter; Schleicher & Schuell Co., Keene, N.H.) were placed on quadrants of the inoculated plates and immediately saturated with 10 μl of saliva or other test sample. Zones of growth inhibition were measured after 48 h of incubation at 37°C in 5% CO_2.

Measurement of starch hydrolysis. After zones of inhibition were measured, plates were washed free of cells with distilled water and flooded with developer to detect enzymatic activity. Gram iodine was used for GC-FC-based media, and the absence of the blue-black iodine-starch reaction around the disk indicated enzymatic hydrolysis of the starch. Charcoal-containing media were flooded with 95% ethanol and incubated at 4°C to precipitate the starch. Zones of clearing around the disks indicated starch hydrolysis.

Treatment of saliva. Parotid saliva was subjected to various treatments to eliminate or isolate the inhibitor.

(i) Heat treatment. Saliva was heated for 30 min at 56, 70, or 80°C.

(ii) Enzymatic digestion. Saliva was incubated with the following enzymes: neuraminidase for 1.5 h at 37°C (50 μg/ml in 0.1 M acetate buffer, pH 5.0), protease type VIII for 3 h at 37°C (80 μg/ml in 0.2 M Tris-hydrochloride buffer, pH 7.6), protease type V for 3 h at 37°C (400 μg/ml in 0.2 M Tris-hydrochloride buffer, pH 7.6), proteinase K for 3 h at 52°C (200 μg/ml in 0.1 M phosphate buffer, pH 7.0), and trypsin for 1.5 h at 37°C (300 μg/ml in 0.05 M Tris-hydrochloride buffer, pH 7.6, containing 2 mM $CaCl_2$).

(iii) Absorption. Dowex 50 (cation-exchange resin) and Dowex 1 (anion-exchange resin) were added to saliva (2.5 parts to 1) and incubated for 15 min. Dowex resin was then removed by centrifugation, and the saliva was filter sterilized. Activated charcoal was tested in a similar manner, except that the charcoal was removed by filtration.

(iv) Ammonium sulfate precipitation. Salivary proteins were precipitated by the dropwise addition of saturated ammonium sulfate with gentle stirring on ice to achieve a final concentration of 50% $(NH_4)_2SO_4$. The precipitate was washed with 50% $(NH_4)_2SO_4$ and then dissolved in mineral salts solution (NaCl, 4.3 g/liter; KCl, 0.42 g/liter; $MgCl_2$, 0.1 g/liter; $CaCl_2$, 0.24 g/liter) to the original volume. The dissolved precipitate and the supernatant were dialyzed overnight against mineral salts solution and then filter sterilized.

(v) Ultrafiltration. Saliva (diluted 1:20 with distilled water) was fractionated on a 200,000-molecular-weight cutoff membrane ultrafiltration disk (Spectra/por UF Discs; Spectrum Medical Industries, Inc., New York, N.Y.). The samples were filtered until the retentate was reduced to its original volume. The retentate was sterilized by filtration.

Methanol extraction of starch. Corn starch (Sigma) or soluble starch (Difco) was soaked 10 times in 2 volumes of methanol (Fisher Scientific Co., Pittsburgh, Pa.) over a period of several days. After air drying at room temperature, the methanol-extracted product replaced starch in the yeast extract-based medium. The methanol washes were collected, evapo-

rated to dryness at room temperature, and reconstituted with 0.5 ml of methanol. A 10-fold dilution of the extract in distilled water was sterilized by autoclaving and used in a well plate assay.

Well plate assay. Wells (5-mm diameter) were cut into agar before inoculation of the media with cell suspension as for the disk assay. Starch extract (40 μl) was added immediately to wells, and plates were incubated right side up. After 48 h at 37°C in 5% CO_2, zones of inhibition were measured.

RESULTS

Inhibition by saliva and α-amylase. The standard assay developed for detecting and quantitating the inhibitory effect of saliva was a disk diffusion procedure on GC-FC agar plates. The average zone size observed with human parotid saliva is shown in Table 1. Similar inhibition could be demonstrated with guinea pig and mouse saliva, although sera from the species tested were not inhibitory. Our previous data (see above) indicated that this inhibition could not be reproduced with any of the traditional mucosal defense factors (i.e., lactoferrin, lysozyme, secretory immunoglobulin A, lactoperoxidase system), but could be duplicated with α-amylase. As shown in Table 1, α-amylases from human saliva, porcine pancreas, bacteria, and fungi all elicited similar zones of inhibition.

Demonstration of amylase-inhibitory activity in saliva. To more convincingly demonstrate that the inhibition observed with saliva was actually due to inherent amylase activity, parotid saliva was treated in various ways to eliminate or isolate the inhibitor. After treatment, the preparations were then tested for both inhibitory and enzyme activity (starch hydrolysis). After treatment, growth inhibition always correlated with starch hydrolysis (Table 2). Both enzyme activity and growth inhibition were sensitive to heating at 80°C (but not 56°C) for 30 min, and to proteinase K treatment (but resistant to the other enzymes tested), absorbed by Dowex 50 cation-exchange resin, precipitated by 50% ammonium sulfate, and retained on ultrafiltration through a 200,000-molecular-weight-cutoff filter. The fact that the enzyme and inhibitory activities were affected by the same treatments (plus the data in Table 1) suggests that the growth inhibition observed for saliva requires the participation of inherent α-amylase activity. The apparent high molecular weight may be due to amylase complexing with itself or other salivary glycoproteins.

Sensitivity of different strains of *L. pneumophila*. The data described above were all ob-

TABLE 1. Inhibitory activity of saliva, serum, and α-amylases on growth of *L. pneumophila* Knoxville-1

Sample	Zone of inhibition (mm^2)[a]
Human saliva	317[c]
Human serum	0
Human salivary α-amylase[b]	280
Guinea pig saliva	150
Guinea pig serum	0
Mouse saliva	163
Mouse serum	0
Bovine pancreatic α-amylase[b]	390
Bacterial (*Bacillus subtilis*) α-amylase[b]	240
Fungal (*Aspergillus oryzae*) α-amylase[b]	100

[a] Area of growth inhibition after 48 h at 37°C in 5% CO_2.

[b] Human salivary amylase was tested at a concentration of 3 mg/ml in sterile distilled water containing 1 mM $CaCl_2$; all other α-amylases were tested at a concentration of 1 mg/ml in sterile distilled water.

[c] The results are the average of the duplicate zones of inhibition from seven different saliva samples.

TABLE 2. Characterization of inhibitor in saliva[a]

Treatment	Growth inhibition[b]	Starch hydrolysis[c]
Untreated	+	+
Heat treatment for 30 min		
56°C	+	+
80°C	−	−
Enzymatic digestion		
Neuraminidase	+	NT
Trypsin	+	NT
Protease type V	+	NT
Protease type VIII	+	NT
Proteinase K	−	−
Absorption		
Charcoal	+	NT
Dowex 1	+	NT
Dowex 50	−	−
Precipitation with 50% ammonium sulfate		
Supernatant	−	−
Precipitate	+	+
Ultrafiltration (200,000 Molecular weight) retentate	+	+

[a] Saliva samples were tested by the disk assay on GC-FC agar.

[b] +, Sample able to produce zone of growth inhibition; −, sample unable to inhibit growth.

[c] +, Sample able to produce starch-free zone; −, sample unable to produce starch-free zone; NT, not tested.

TABLE 3. Sensitivities of *L. pneumophila* strains to saliva and amylase

Strain (Serogroup)	Zone of inhibition (mm^2)[a]
Laboratory-adapted strains[b]	
Bellingham-1 (1)	137 ± 37 (75–239)
California-1 (1)	103 ± 15 (75–138)
Knoxville-1 (1)	158 ± 13 (133–177)
Philadelphia-2 (1)	314 ± 27 (245–377)
Togus-1 (2)	302 ± 30 (230–359)
Bloomington-2 (3)	216 ± 23 (150–245)
Los Angeles-1 (4)	170 ± 26 (115–239)
Dallas 1E (5)	445 ± 64 (276–577)
Chicago-2 (6)	357 ± 43 (230–425)
Animal-passaged strains[c]	
Knoxville-1^v	154 ± 11 (133–165)
Los Angeles -1^v	171 ± 3 (165–177)
Chicago-2^v	299 ± 9 (284–314)

[a] Mean of area ± standard error (range) of growth inhibition after 48 h of incubation at 37°C in 5% CO_2.
[b] Strains were tested in duplicate with four saliva samples.
[c] Strains were tested in duplicate with two saliva samples and with bacterial amylase.

tained with the Knoxville-1 strain of *L. pneumophila*. The results in Table 3 indicate that this phenomenon is common to all of the strains examined, although there was a reproducible difference in sensitivity between strains. In addition, the Knoxville-1 (laboratory-adapted) and Knoxville-1^v (animal-passed) strains showed the same degree of sensitivity. Similar results were observed with animal-passed strains of Los Angeles-1 and Chicago-2.

Mechanism of amylase-induced growth inhibition. Since the disk diffusion assay was performed on an agar medium (GC-FC) containing starch, it was necessary to distinguish between a possible direct action of amylase on the bacteria and an indirect effect secondary to the enzymatic action on the medium. To investigate this question, the disk assay procedure was modified to allow diffusion of saliva into the medium, followed by heat inactivation (100°C, 1 h) of the enzyme before inoculation with bacteria. The criterion for effective heat treatment was a zone of starch hydrolysis that did not increase in area during 48 h of continued incubation. Plates that had been heat treated (no active amylase) still had zones of inhibition (Table 4). As expected, these zones were smaller than those on plates that were not heated, since the amylase on the latter plates continued to diffuse in the medium, giving larger zones of both starch hydrolysis and inhibition. These results indicated that the growth inhibition was a result of the amylase interacting with the medium (i.e., creating a toxic environment), rather than a direct effect of amylase on the bacteria.

Abrogation of the amylase effect. Modifications of GC-FC medium (proteose-peptone based) were examined to more definitively characterize the mechanisms of inhibition by amylase (Table 5). The addition of charcoal or bovine serum albumin blocked growth inhibition, but had no effect on the hydrolysis of starch in the medium. Inhibition could also be demonstrated on a buffered yeast extract-based medium containing 0.1% corn starch, and the addition of charcoal to this medium abrogated the growth inhibition. These results suggest that the hydrolysis of starch exposes or creates an inhibitory substance that can be bound or in some way blocked by the addition of charcoal or bovine serum albumin.

Identification of toxic media components. With *N. gonorrhoeae*, the toxic factor appears to reside in the agar (8, 11), and the use of purified agar permitted growth without the necessity for starch (9). With *L. pneumophila* we observed that when Noble agar (Difco) was substituted for the Bacto-Agar in the GC-FC or buffered yeast extract-based medium, growth could be obtained without starch, and neither amylase nor saliva had any inhibitory effect (Table 6). However, zones of inhibition were once again observed when the media were prepared with corn starch, but not when the media were prepared with soluble starch. This interesting finding suggested that growth inhibition was the result of two inhibitory factors, one in the agar that could be eliminated by using a more purified product and another contributed by the starch component of the medium.

To eliminate the toxic component, corn starch was purified by methanol extraction before the addition to the basal medium. Growth inhibition

TABLE 4. Heat inactivation of saliva on GC-FC medium

Disk-sample	Heat treatment[a]	Inhibition zone (mm^2)[b]	Hydrolyzed starch zone (mm^2)[c]
Saliva	–	1,030	5,675
Saliva	+	392	563
Water	+	0	0

[a] Conditions for complete inactivation were predetermined. The criterion for effective heat treatment was a zone of hydrolysis that did not increase after extended incubation.
[b] Mean of area of zone of inhibition after 18 h of pretreatment of medium and 48 h of incubation after inoculation (n = 4).
[c] Mean of area of starch hydrolysis detected by iodine reaction. The total area of glass petri plates is 5,675 mm^2.

induced by amylase activity was eliminated on buffered yeast extract-based agar medium prepared with Noble agar and methanol-extracted corn starch. In addition, the extract of corn starch, but not soluble starch, inhibited growth of *L. pneumophila* in a well plate assay independent of amylase action.

DISCUSSION

The present studies indicate that the growth on solid media of all strains of *L. pneumophila* tested was inhibited by the presence of α-amylase (either salivary, pancreatic, bacterial, or fungal), extending the previously reported observation of an amylase effect on *N. gonorrhoeae* (13, 14). Both organisms are sensitive to toxic components of media, necessitating the addition of factors such as starch, bovine serum albumin, or charcoal to neutralize the inhibitory effect (17) (Table 6). The inhibition of both species by amylase was observed only in the presence of starch.

L. pneumophila will not grow in the absence of starch on media containing Bacto-Agar, but will grow when Noble agar is substituted. Starch apparently neutralizes the toxic component(s) of Bacto-Agar that are unmasked by starch hydrolysis. This is supported by the observation that heat inactivation of amylase after hydrolysis did not prevent inhibition (Table 4). The importance of starch is suggested by the fact that neither amylase or saliva is inhibitory on media containing Noble agar in the absence of starch. Interestingly, amylase and saliva were both inhibitory on Noble agar when corn starch, but not soluble starch, was added. This inhibition could be eliminated by methanol extraction of the corn starch and the inhibitory component demonstrated in the extract. These studies suggest that both Bacto-Agar and corn starch contain inhibitory substances for *L. pneumophila* that are neutralized by intact starch, suggesting an indirect effect of amylase on *L. pneumophila*. The conditions of inhibition for *N. gonorrhoeae* by amylase are identical to those for *L. pneumophila* and therefore raise some question as to the previous interpretation of a direct amylase effect on solid media.

Mellersh et al. (14) also examined the effects of amylase on the gonococcus in liquid medium. This medium contained soluble starch, which, based on our observations (Table 6), may not have been toxic. These studies suggested that amylase induced osmotically fragile, cell wall-defective variants of gonococcus. Mellersh et al. interpreted the growth inhibition observed in the agar diffusion assay to be due to the same mechanism. In preliminary investigations in our laboratory we have tested the effects of amylase on *L. pneumophila* in starch-free liquid medium. Viability was determined on charcoal-containing, buffered yeast extract-based medium to eliminate any inhibition due to medium components. There was a time-dependent loss in recoverable colonies that was enzyme dependent and apparently independent of toxic medium components. Current studies are directed at

TABLE 5. Comparison of the effects of various media components on the inhibition of growth of *L. pneumophila* by α-amylase

Medium[a]	Growth inhibition[b]	Starch hydrolysis[c]
Proteose peptone agar base		
+ Starch	+	+
+ Starch and charcoal	−	+
+ Starch and BSA	−	+
+ BSA	−	NT
Yeast-extract agar base		
+ Starch	+	+
+ Starch and charcoal	−	+
+ Charcoal	−	NT

[a] Corn starch (0.1%, wt/vol; Sigma) and activated charcoal (0.15%, wt/vol; Sigma) were added to the basal media before autoclaving. Bovine serum albumin was filter sterilized separately and added to the tempered basal medium after autoclaving to achieve a final concentration of 0.1% (wt/vol).

[b] +, Sample able to produce a zone of growth inhibition; −, sample unable to inhibit growth.

[c] +, Sample able to produce a starch-free zone; NT, not tested since there was no starch in the medium.

TABLE 6. Effect of α-amylase on growth of *L. pneumophila* Knoxville-1 on media containing Noble agar

Medium[a]	Growth on the medium[b]	Zone of inhibition[c]	Starch hydrolysis[d]
Proteose peptone-Noble agar:			
− Starch	+	−	NT
+ Corn starch	+	+	+
Yeast extract-Noble agar:			
− Starch	+	−	NT
+ Corn starch	+	+	+
+ Soluble starch	+	−	+

[a] Noble agar was substituted for Bacto-Agar at equal concentration; 0.1% (wt/vol) corn starch (Sigma) or soluble starch (Difco) was added before autoclaving.

[b] Relative ability of the medium to support the growth of *L. pneumophila*; +, good growth.

[c] +, Zone of growth inhibition; −, no zone of inhibition. The disk contained 10 μg of bacterial α-amylase.

[d] +, Sample able to produce a starch-free zone; NT, not tested since there was no starch in the medium.

characterizing the mechanism of action of this inhibition and distinguishing it from the starch-dependent amylase effect.

In conclusion, these data indicate that toxic effects of agar and starch on *L. pneumophila* can be unmasked by starch hydrolysis. This indirect effect seems to be the major contributor to amylase inhibition on solid media, as growth is not affected by amylase when nontoxic preparations of agar and starch are used. These media-dependent effects must be eliminated before attempting to characterize any direct effects of amylase or other components of mucosal secretions on *L. pneumophila* and probably *N. gonorrhoeae*.

Regardless of the mechanism(s) of action of amylase on *L. pneumophila*, its effects may explain the difficulty in isolation and the need for charcoal in the medium for primary culture of mucosal specimens. Amylase produced by other pharyngeal flora may also act to suppress *L. pneumophila*, although amylase cannot explain the inhibition by normal flora observed on charcoal containing media (3). If there is any validity to the bactericidal assays and the gonococcal data (13, 14), then amylase may play some role in the host defense of mucosal surfaces. The more traditional mucosal defense factors may serve as a necessary compromising influence to render *Legionella* susceptible to amylase. The possibility of such a synergism is currently being investigated by determining the effects of lactoferrin, lysozyme, lactoperoxidase, and secretory immunoglobulin A on *L. pneumophila* both singly and in combination with amylase.

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