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Hyaluronic Acid Capsule: Strategy for Oxygen Resistance in Group A Streptococci

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Unencapsulated variants of encapsulated, M-protein-positive group A streptococci are oxygen sensitive and secrete inhibitory concentrations of hydrogen peroxide when grown in aerated broth cultures. The organisms were equally sensitive to hydrogen peroxide, and neither exhibited catalase or peroxidase activity, suggesting that differences in oxygen sensitivity reflect dissimilarity in oxygen uptake. The encapsulated parental culture was found to grow in aggregates that take up oxygen more slowly than unencapsulated, oxygen-sensitive derivatives. Moreover, the latter grow in an unaggregated, homogenous suspension. The enzyme hyaluronidase was able to disrupt aggregates of the encapsulated strain, increase the rate that these cells take up oxygen, and cause the accumulation of toxic concentrations of hydrogen peroxide earlier in their growth cycle. The evidence presented shows that the aggregation of streptococcal cells by their hyaluronic acid capsule provides this organism with a novel means to avoid self-destruction by oxygen metabolites—cells are shielded from oxygen. The reduced surface-to-volume ratio and limited diffusion of oxygen into the interior of aggregates are proposed as the protective mechanism.

Various theories have been proposed to account for the extreme toxicity of oxygen to many anaerobic and facultative anaerobic bacteria (20). Recent proposals have implicated the products of the univalent reduction of molecular oxygen, very reactive superoxide anions, singlet oxygen, and hydroxyl free radicals as the lethal agents (3, 8, 19, 20). Moreover, Fridovich and his colleagues have demonstrated the hazardous potential of these molecules and have provided convincing evidence that the enzyme superoxide dismutase protects some facultative and obligate aerobes by scavenging these free radicals to form hydrogen peroxide (3, 8, 19).

As lactic acid bacteria, the group A streptococci gain energy by an anaerobic metabolism and lack the enzyme catalase. Although aerotolerant, it is generally recognized that most cultures of this species grow faster and yield more cells when incubated anaerobically or in an environment enriched for carbon dioxide. Antecedents to streptococcal pharyngitis are the adherence of streptococci to pharyngeal cells and their subsequent survival and multiplication in a highly aerobic environment, the nasopharynx. Therefore, this organism is faced with the need to either detoxify the products of oxygen metabolism or avoid the uptake of this potentially lethal molecule. Detoxification would be expected to play a major role in the fact that these organisms are aerotolerant. Streptococci, including group A bacteria, possess superoxide dismutase (3), and many strains are known to release ample quantities of hydrogen peroxide (18). It seems reasonable, however, that a detoxification pathway that releases hydrogen peroxide as an end product, a highly reactive and toxic chemical in itself, would have limitations and would require that catalase-negative species, in particular, develop other means to avoid self-destruction by oxygen metabolism.

The outer envelope of the group A streptococcus is a mosaic of proteins, the M and T antigens, and polysaccharides, the group A carbohydrate and lipoteichoic acid complexed in unknown ways to the peptidoglycan shell or cytoplasmic membrane; a hyaluronic acid capsule is also loosely attached in many strains (13, 27). When comparing the growth of virulent encapsulated strains to unencapsulated avirulent variants, it was observed that the latter grew poorly in the presence of atmospheric oxygen. This intriguing observation was pursued further, and the results to be reported here indicate that hyaluronic acid provides this encapsulated bacterium with a protective shield to oxygen.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The four strains of group A streptococci used in these

studies were: strain CS24 (T-12, M-12), originally designated K56 and obtained from E. Kjems, and three variants from this strain: CS46, a spontaneous glossy, M^- isolate; strain CS44, an A25 phage-resistant isolate; and strain CS64, a spontaneous, glossy, M^- variant of strain CS44 (4). The strains were routinely T typed by the agglutination method of Griffith (10) and M typed by the capillary precipitin method (8, 26). For typing procedures and phagocytosis experiments, the bacteria were grown in Todd-Hewitt broth (Difco) at 37°C. Cultures were tested for resistance to phagocytosis with human blood by methods previously described (4, 5). For growth curves, hydrogen peroxide production, aggregation, and oxygen uptake studies, bacteria were grown at 37°C in serum-supplemented brain heart infusion media (SBHI) described by Malke (18). Stock cultures were bacteria grown to log phase in no. 1 broth (25) at 37°C, resuspended in fresh no. 1 broth with 15% glycerol, and frozen in liquid nitrogen.

Growth curves and hydrogen peroxide measurements. The bacteria were grown overnight at 37°C in stationary SBHI broth, then inoculated into aerated broth (shaken overnight at 37°C before inoculation), and incubated at 37°C with vigorous aeration in a New Brunswick shaker water bath. Hourly samples were removed for viable counts on tryptose blood agar (Difco), and hydrogen peroxide was determined by the method of Emiliani and Riera (6).

When cells were to be grown in the presence of trypsin, the inoculum was first digested with trypsin (Difco, 1:250) at a final concentration of 5 mg/ml for 45 min at 37°C before they were inoculated into aerated SBHI broth containing the same concentration of trypsin. At 4 h, more trypsin was added to give a final concentration of 4 mg/ml. In a similar manner, cells were digested with bovine testis hyaluronidase. Hyaluronidase was added to the overnight inoculum to a final concentration of 5.7 mg/ml, and the mixture was incubated for 1 h at 37°C before it was added to fresh aerated SBHI broth also containing hyaluronidase (2.8 mg/ml). Hyaluronidase was again added at 3.5 h, resulting in a final total concentration of 3.7 mg/ml.

Protein and oxygen determinations. Protein determinations were performed on washed whole cells. Cells were suspended in cold 10% trichloroacetic acid for 30 min. The resulting precipitate was dissolved in 1.0 M NaOH and ultimately brought to a final volume of 2 ml with distilled water. Samples of this extract were assayed for protein by the Lowry method (15).

The rate of oxygen uptake by streptococcal cells was determined by observing the disappearance of oxygen from cell suspensions with a Yellow Springs oxygen electrode and analyzer. Eighteen-hour cells (0.4 ml) were injected into a closed chamber which contained 3 ml of 0.05 M phosphate buffer, pH 7.8, and 24 mM glucose. Before cells were injected, the buffer in the chamber was mixed at 37°C until the oxygen concentration was equilibrated. Upon injection of cells, the decrease in oxygen concentration was monitored until it no longer changed.

Scanning electron microscopy. Bacteria were grown for 18 h in Todd-Hewitt broth, washed several times with phosphate-buffered saline (10 mM sodium phosphate, 0.145 M sodium chloride, pH 7.2), fixed with glutaraldehyde in 0.1 M cacodylate buffer, and finally fixed with osmium tetraoxide by the method of Malick (17). Preparations were dehydrated by a graduated series of ethanol washes and then air dried with propylene oxide. The microscope employed was an ETEC Autoscan.

RESULTS

The M^+ and M^- streptococcal strains employed in these experiments exhibited the previously described mucoid-mat and glossy colony morphology, respectively. To confirm that this dissimilarity depended on hyaluronic acid, both the entire cultures and the culture supernatant fluids were assayed for hyaluronic acid by the method of Tolksdorf et al. (12, 24). Six-hour cultures of the M^+ encapsulated strain, strain CS44, contained in total 152 g of hyaluronic acid per ml, whereas no hyaluronic acid could be detected in identical cultures of the M^- unencapsulated cells, strain CS64. Controls, hyaluronidase-digested cells from each strain, had no detectable hyaluronic acid, verifying the specificity of the assay. The encapsulation of strain CS44 was also confirmed by microscopic observation of cells stained with India ink.

Colonies of the unencapsulated (Cap^-), M-protein-deficient (M^-) bacteria, strains CS46 and CS64, were barely visible on blood agar plates incubated aerobically, in contrast to Cap^+ M^+ parent strains, CS24 and CS44. In an anaerobic environment (GasPak anaerobic system, BBL Microbiology Systems), both Cap^+ M^+ and Cap^- M^- strains formed colonies of normal size. Furthermore, the unencapsulated strains CS46 and CS64 produced a labile extracellular substance which inhibited the growth of various other strains of group A streptococci, including themselves and their parental strains. The inhibitory substance was detected by spotting the Cap^- M^- cells on indicator lawns (Fig. 1). Figure 1A shows zones of growth inhibition when cultures of strains CS64 or CS46 were spotted on strain CS24 as an indicator. Encapsulated cultures, strains CS24 and CS44, failed to show inhibitory activity. If catalase were incorporated into the agar medium (Fig. 1B), the inhibitory activity was not apparent, and as expected the inhibitory effects of spotted H_2O_2 were also destroyed. The effect of catalase and the lack of inhibition when indicator plates were incubated anaerobically suggested that the Cap^- M^- variants secrete bactericidal quantities of H_2O_2.

The difference between these encapsulated and unencapsulated strains with respect to oxygen sensitivity was even more dramatic when their growth rates were compared in aerated

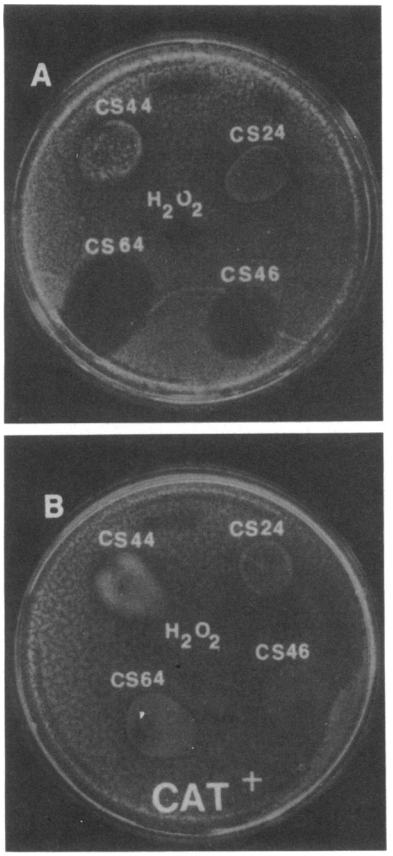

FIG. 1. *Bactericidal activity of unencapsulated strains. Cultures grown for 18 h in SBHI at 37°C were spotted on SBHI agar plates previously spread with 18-h cultures of strain CS24 (A). (B) is the same with the exception that the agar contained 580 U of bovine liver catalase (Sigma) per ml. A drop of hydrogen peroxide (3%) was spotted in the center of each plate as a control. After drying, the agar plates were incubated for 18 h at 37°C.*

broth cultures (Fig. 2). Under these conditions, strain CS44 grew at a reasonable rate, a doubling time of approximately 60 min; the unencapsulated culture, strain CS64, underwent only one division in 6 h. If aeration were continued for 18 h, strain CS64 cultures could be completely sterilized. This strain, however, was able to grow at the same rate as strain CS44, with the distinction of a 2-h lag in growth, when catalase was incorporated into the aerated broth cultures. This again indicates that H_2O_2 or its precursor, superoxide, was responsible for the deleterious effects of aeration.

In support of the above contention was the striking difference in the amount of H_2O_2 released by these cultures during growth (Fig. 3). Strain CS44 cultures produced no H_2O_2 until early stationary phase (6 to 7 h), at which time H_2O_2 began to accumulate very rapidly. On the other hand, cultures of strain CS64 exhibited a burst of H_2O_2 synthesis that began 2 h after inoculation and continued throughout the incubation period. After 18 h of incubation, both cultures had accumulated appreciable quantities of H_2O_2, accompanied by a dramatic drop in viability (data not shown). This drop in viability reflected a 4-log increase in the concentration of H_2O_2 per colony-forming unit (Fig. 3). The concentration of H_2O_2 is plotted both in micromoles per milliliter and micromoles per colony-forming unit to more fully exemplify the vast difference between these strains.

The failure of strain CS44 to excrete H_2O_2 during log-phase growth and its ability to multiply in an aerated environment could be explained if these cells were better suited than strain CS64 cells to resist the cidal activity of hydrogen peroxide. This explanation appeared

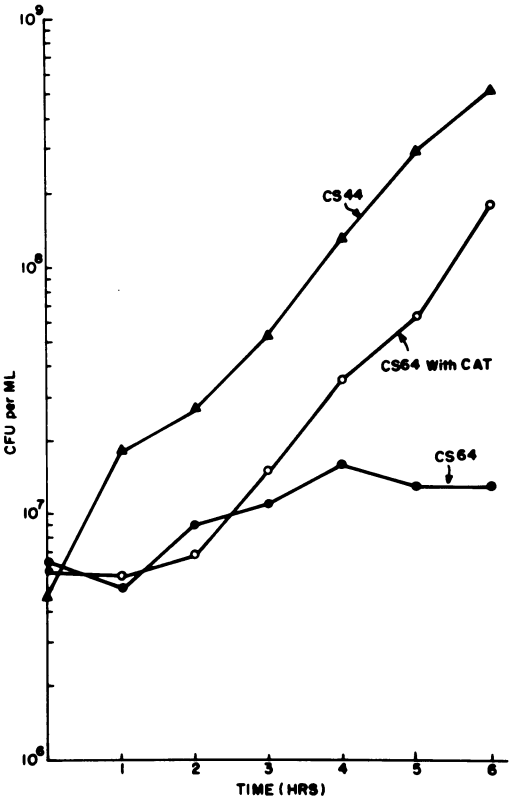

FIG. 2. *Growth of encapsulated and unencapsulated strains under aerated growth conditions. Eighteen-hour cultures were inoculated into aerated SBHI broth. The culture designated CAT contained catalase at a concentration of 540 U/ml. CFU, Colony-forming units.*

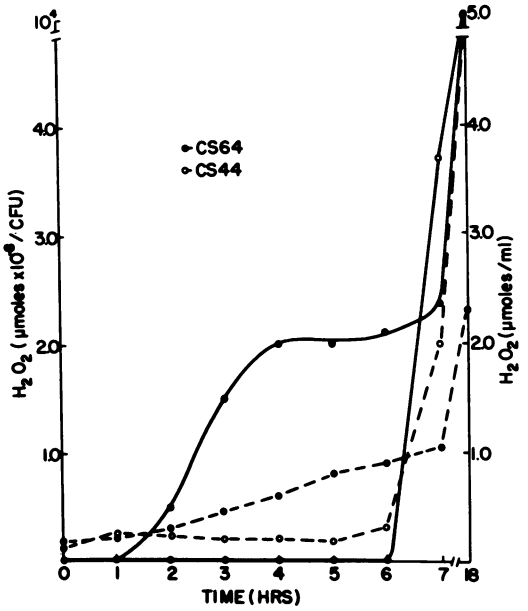

FIG. 3. *Production of hydrogen peroxide. Cultures were inoculated into aerated SBHI as described for Fig. 2. Samples were removed, and their hydrogen peroxide content was determined (6). The concentration is plotted as 10^{-8} μmol per colony-forming unit (——) and micromoles per milliliter (-----).*

unlikely when their death rates were compared. Both strains were killed at equal rates when cells were suspended in buffer or medium containing H_2O_2 (30 μmol/ml) (Fig. 4). Moreover, neither whole cells nor culture supernatant fluids from either strain degraded H_2O_2 at a significant rate. Thus, peroxidase or catalase activity specific to the Cap^+ M^+ strain does not account for its relative resistance to O_2.

Many cultures were microscopically examined by dark-field phase microscopy. The observation that encapsulated cells grew in large aggregates resembling microcolonies suggested to us another explanation for the oxygen resistance of strain CS44. The unencapsulated cells, strain CS64, grew primarily in a homogeneous suspension of single chains. By scanning electron microscopy (Fig. 5) strain CS44 cells were observed to be present in disorganized clumps covered by a gelatinous layer, presumably hyaluronic acid. Although a distribution was not determined quantitatively, clumps appeared to be fairly uniform in size.

Aggregation could create a localized microaerophilic environment and does, in fact, cause cells to settle to the bottom of the culture tube where the O_2 concentration is reduced. The degree of aggregation could be simply estimated by determining the rate at which stationary suspensions of cells sedimented to the bottom of a Klett tube. Encapsulated cells, strain CS44, settled out of suspension very rapidly, indicating their clumped nature. These aggregates could be thoroughly abolished by either trypsin or hyaluronidase digestion preventing their subsequent sedimentation (Fig. 6). Examination of both hyaluronidase- and trypsin-treated cells by phase-contrast microscopy confirmed this disaggregated state. Furthermore, cells genetically devoid of hyaluronic acid and M protein, strain CS64, remained indefinitely in suspension (Fig. 6). These results indicated that the aggregated state requires both a protein (possibly M protein) and the polysaccharide capsule. Thus, group A streptococci may be analogous to *Streptococcus mutans* in which dextran anchored to the cell surface by a protein is required for aggregation (9).

If aggregation, dependent in part on intact

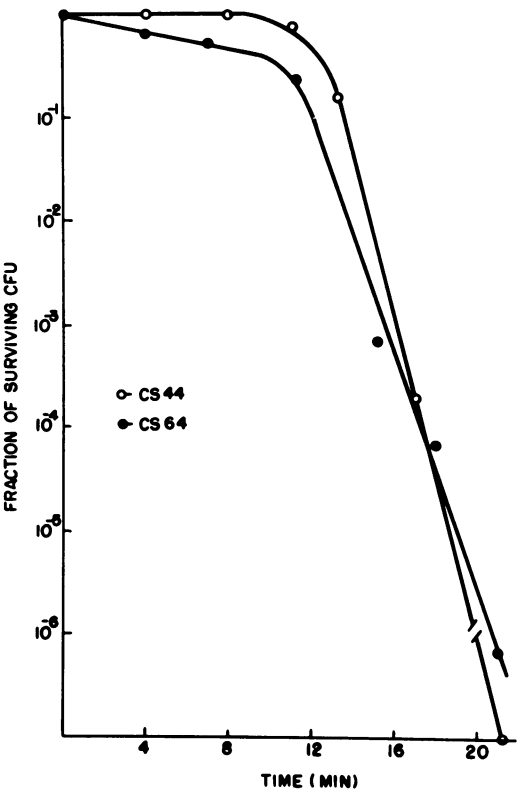

FIG. 4. *Sensitivity of encapsulated and unencapsulated strains to hydrogen peroxide. Log-phase cultures, grown for 2.5 h at 37°C in SBHI broth, were resuspended in phosphate-bufferd saline. At zero time hydrogen peroxide was added to a final concentration of 0.1%. Samples were withdrawn at the indicated time intervals, diluted in phosphate-buffered saline containing catalase, 630 U/ml, and plated for viable counts.*

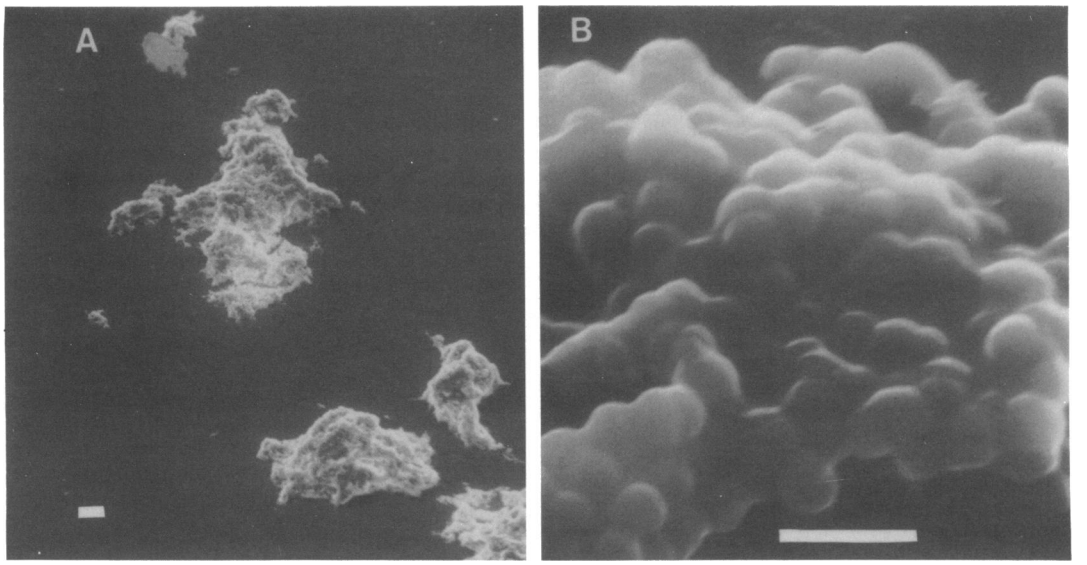

FIG. 5. *Scanning electron micrographs of strain CS44 aggregates. Washed 18-h cultures of strain CS44 were fixed, dried, and scanned as described in the text. The white bar equals 5 μm (A) and 0.5 μm (B).*

hyaluronic acid polymer, is crucial for the O_2 resistance of strain CS44, then treatment of these cells with hyaluronidase should cause them to release H_2O_2 earlier in their growth cycle. Table 1 shows the effects of hyaluronidase, trypsin, and nonaerated growth conditions on H_2O_2 production. Representative data from separate experiments are shown here. Untreated aerated cultures were included in each experiment for comparison to those enzymatically digested. No H_2O_2 above the blank level of uninoculated broth was produced by nonaerated cultures of strain CS44; however, measurable H_2O_2 was produced by strain CS64 under these conditions. Digestion of strain CS44 cells with hyaluronidase caused them to accumulate detectable levels of H_2O_2 2 h earlier than undigested cells. Moreover, in hyaluronidase-treated cultures of strain CS44, the increased rate of H_2O_2 production was accompanied by the premature cessation of growth and even cell death. Although we expected trypsin to have a similar impact on H_2O_2 synthesis, it was surprising to find that this enzyme totally inhibited the secretion of H_2O_2 by both cultures; yet, this treatment did not save the unencapsulated cells from oxygen-induced death (unpublished data). The possibility that trypsin was contaminated with peroxidase or catalase activity was excluded by incubating trypsin with H_2O_2 under the conditions employed in Table 1, and no change in the H_2O_2 concentration was observed. This is interpreted to mean that oxygen precursors to H_2O_2 or some other toxic metabolite induced by aeration rather than extracellular H_2O_2 contributes to the death of M^- Cap^- cells, a finding in harmony with the conclusion of other investigators (8, 19, 20). An explanation for the inhibitory effect of trypsin on H_2O_2 accumulation was beyond the scope of the present study.

The above results suggest that the physical aggregation of streptococcal cells by hyaluronic acid capsular material creates a barrier to O_2 and that the absence of this barrier, which in part distinguishes Cap^- M^- from Cap^+ M^+ cells, could explain the oxygen sensitivity of the former. If this were true, then strain CS64 should take up O_2 more rapidly than strain CS44, which is highly encapsulated. This possibility was tested by measuring with an oxygen electrode and analyzer the rate of O_2 utilization by cells suspended in buffer. The initial rates (first 2 min) are plotted as a function of protein concentration of Fig. 7. During this initial time period, O_2 uptake was a linear function of time and rates were directly proportional to the protein concentration. A steady-state condition with very little change in the external concentration of O_2 was attained when the O_2 concentration reached 0.05 μmol/ml and was independent of the protein concentration.

As anticipated, strain CS64 cells took up O_2 two to three times faster than strain CS44 cells (Fig. 7), depending on the protein concentration. Moreover, digestion of CS44 cells with hyaluronidase dramatically increased the rate at which these cells took up O_2; in fact, hyaluronidase-treated CS44 cells were equivalent to CS64 cells

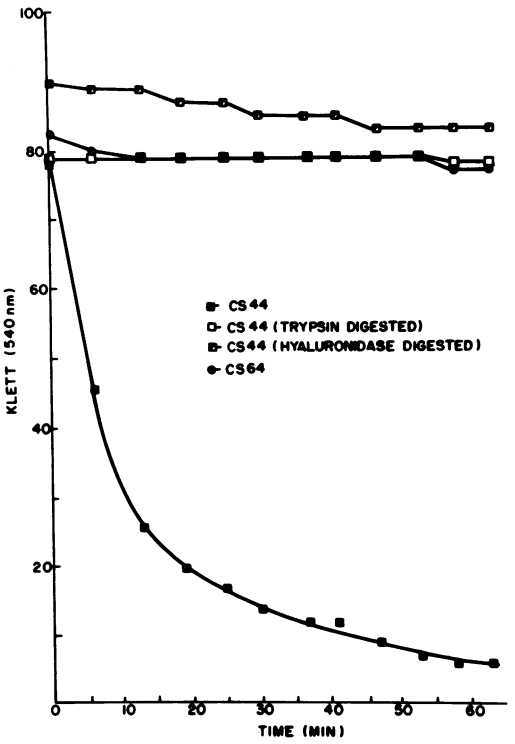

FIG. 6. *Sedimentation of encapsulated and unencapsulated streptococci. Eighteen-hour cells were resuspended in a Klett tube containing phosphate-buffered saline, and Klett readings at 540 nm were made in a Klett-Summerson colorimeter at the indicated times. Cell suspensions were divided into three portions; one portion was left untreated, one portion was digested with trypsin (8 mg/ml), and another portion was digested with hyaluronidase (6 mg/ml) all for 1 h at 37°C.*

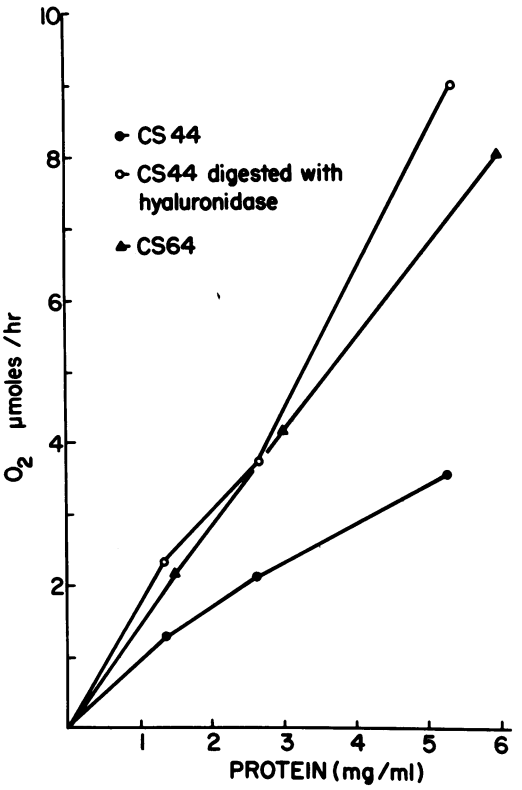

FIG. 7. *Oxygen uptake by encapsulated and unencapsulated streptococci. Eighteen-hour cultures were concentrated 20-fold in culture supernatant, and at zero time 0.4 ml of various dilutions of the concentrate was injected into 3 ml of phosphate buffer containing glucose equilibrated at 37°C in a closed chamber containing a Yellow Spring oxygen electrode. Cells to be digested with hyaluronidase were grown for 18 h in medium containing hyaluronidase (0.67 mg/ml) and then subjected to an additional treatment for 1 h with fresh hyaluronidase (0.33 mg/ml) just before each experiment.*

TABLE 1. *Influence of aeration, trypsin, and hyaluronidase on H_2O_2 accumulation*

Strain/culture conditions	H_2O_2 (μmol/ml) at postinoculation h:		
	5	6	7
Strain CS44			
Nonaerated	0.18	0.13	0.15
Aerated	0.20	0.25	5.71
Hyaluronidase digested[a]	0.67	5.65	9.25
Trypsin digested	0.12	0.28	0.21
Strain CS64			
Nonaerated	0.47	ND[b]	0.67
Aerated	0.83	1.23	1.66
Hyaluronidase digested	ND	ND	ND
Trypsin digested	0.23	0.25	0.28

[a] Conditions used for the digestion of cells with hyaluronidase and trypsin are described in the text.
[b] ND, Not done.

in this regard (Fig. 7). Therefore, it was concluded from this experiment that the presence of a hyaluronic acid capsule markedly reduces the potential of this strain of group A streptococcus to take up O_2. Furthermore, the absence of this barrier to O_2, a characteristic of avirulent $Cap^- M^-$ cells, accounts for the extreme sensitivity of these cells to O_2.

DISCUSSION

Anaerobic bacteria can be very sensitive to atmospheric concentrations of oxygen, whereas facultative anaerobic and aerobic organisms are relatively resistant. The latter have evolved various means to overcome the toxic products of oxygen metabolism such as superoxide and other

free radicals. Central to oxygen resistance in both eucaryotic and procaryotic cells is the enzyme superoxide dismutase (19), which is able to scavenge superoxide molecules by converting them to hydrogen peroxide and O_2. Organisms that lack the potential to synthesize this enzyme, including most anaerobes, are exquisitely sensitive to small concentrations of O_2.

Hydrogen peroxide is in itself highly reactive and toxic to living cells (1), and for this reason the capacity of superoxide dismutase to protect cells from oxygen would seem limited by their potential to deal with the resultant hydrogen peroxide. Some species produce peroxidases and catalases that destroy accumulating hydrogen peroxide and thus serve a protective role (20, 22, 23). Other mechanisms of oxygen resistance, such as its exclusion at the cell surface, have received little attention.

Virulent group A streptococcal colonies are mucoid because they produce copious quantities of hyaluronic acid in the form of a gel-like capsule (11, 27). Most strains yield glossy variant colonies which fail to produce hyaluronic acid and the antiphagocytic M protein (12, 27). Often, on passage in animals or in human phagocytic blood, colonies can be retrieved that produce both the M antigen and hyaluronic acid. Although the coordinate synthesis of M protein and capsule can be phenotypically disrupted (27), the genetic relationship of one to the other is presently not understood.

When comparing the growth characteristic of an encapsulated strain to a spontaneous unencapsulated variant, it was discovered that the latter was extremely sensitive to oxygen and produced an inhibitory substance identified as hydrogen peroxide. Other pairs of encapsulated and unencapsulated strains also showed this property to various degrees, but strains CS24, CS44, and their unencapsulated variants showed the most dramatic differences with regard to oxygen sensitivity. The parent encapsulated strain was aerotolerant in aerated broth cultures and only produced detectable hydrogen peroxide during the stationary growth phase. In contrast, the unencapsulated cells produced detectable hydrogen peroxide within 2 h after inoculation into aerated broth, as well as under stationary culture conditions with minimal aeration. Because the two strains were equally sensitive to hydrogen peroxide and neither exhibited catalase or peroxidase activity, we postulated that their dissimilarity with regard to oxygen sensitivity must depend on differences in oxygen uptake, consumption, or both that are reflected by the accumulation of hydrogen peroxide by the oxygen-sensitive, unencapsulated strain. This, in fact, proved to be true; unencapsulated cells took up oxygen at a considerably faster rate than the encapsulated parent cells. This difference in rate was only evident within minutes after the introduction of cells into aerated medium or buffer, suggesting that uptake rather than the metabolic consumption of oxygen was the limiting factor. The hyaluronic acid capsule of the encapsulated parent culture proved to be the direct determinant of oxygen resistance. When the encapsulated cells were digested with the enzyme hyaluronidase, they accumulated toxic concentrations of hydrogen peroxide much earlier in their growth cycle and in separate experiments were found to take up oxygen at a rate equivalent to the unencapsulated aerosensitive strain.

Totally consistent with these results was the observation that the encapsulated bacteria grew in a highly aggregated state that could be dispersed by hyaluronidase. The aggregates appeared uniform in size and were estimated to have a radius of approximately 50 μm. If both aggregates and cocci are assumed to be spherical, the area-to-volume ratio for a coccus can be calculated to be 6.02 $\mu m^2/\mu m^3$, two orders of magnitude greater than that of the aggregates (0.05 $\mu m^2/\mu m^3$). Intuitively, this difference would seem more than adequate to account for the greater rate of oxygen uptake by Cap^- evenly dispersed cells. The influence of particle size on the efficiency of aeration has been described by Phillips (21). He reported that as the radius of a fungal pellet increased, the oxygen concentration within the pellet decreased. At radii greater than 50 μm, the oxygen concentration dropped off precipitously. Secondary to the influence of reduced surface area is the possibility that the envelope of hyaluronic acid polymer directly retards the diffusion of oxygen into cells. This layer can approach 0.5 μm in thickness (unpublished data) and in a growing culture ultimately permeates the entire culture fluid to the extent that the viscosity of the fluid measurably increases (11). In support of this idea, Brown stated that the slime that accumulates during fermentation can severely limit oxygen consumption by growth fungi (3). Unfortunately, we are unable to evaluate the possible direct effect of capsular material on oxygen uptake because diffusion coefficients in related or similar biopolymers are unavailable.

The aerotolerant group A streptococci without the aid of catalase have evolved, until now, an undescribed strategy for survival in an aerobic environment; oxygen is simply excluded from the cell, at least cells at the interior of an encapsulated clump. Hyaluronic acid capsule and a trypsin-sensitive protein are required for cellular

aggregation, and this condition reduces O_2 uptake. In the absence of this barrier, unencapsulated M^- cells produce copious quantities of hydrogen peroxide which along with other products of oxygen metabolism ultimately becomes toxic.

The impact of accumulated hydrogen peroxide on the genetic instability of encapsulation and M protein synthesis is unknown, but could be significant. This toxic by-product is known to be mutagenic, to cause single-strand breaks in DNA and to induce prophage (1, 7, 16). Any one of these mechanisms could alter the composition and quantity of these somatic structures and therefore influence the genetic variability of these organisms. Thus, in addition to resistance to phagocytosis, one more function, oxygen resistance, can be ascribed to the outer envelope of group A streptococci. The importance of this resistance could be exaggerated in vitro; however, it seems reasonable to surmise that this property may facilitate the colonization by this bacterium of the nasopharynx, an extremely aerobic environment.

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