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Intracellular Survival of *Streptococcus pyogenes* in Polymorphonuclear Cells Results in Increased Bacterial Virulence

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It has recently been shown that survival within phagocytic cells constitutes an additional strategy used by *Streptococcus pyogenes* to evade the host defenses. Here we provide evidence that *S. pyogenes* can escape from the phagosome into the cytoplasm of phagocytic cells. Furthermore, intracellular bacteria seem to undergo phenotypic switching that results in much more virulent microorganisms.

Streptococcus pyogenes is an important human pathogen that can cause severe, life-threatening, invasive infections such as soft-tissue infection, sepsis, and streptococcal toxic shock syndrome (3). *S. pyogenes* is generally an extracellular pathogen that can survive and persist within the host by the expression of a broad array of virulence functions directed to circumvent the host immune mechanisms (5, 7, 11, 14). With a murine model of streptococcal infection, it has been shown that an additional strategy used by *S. pyogenes* to circumvent the host defenses is to avoid the killing mechanisms of polymorphonuclear neutrophils (PMNs) and survive within these cells (10). This strategy was clearly demonstrated by the ability of PMNs isolated from infected mice to transfer infection after being inoculated into naïve mice (10). By surviving within phagocytic cells, *S. pyogenes* can also exploit the free-trafficking privileges of these cells within the host to systemically disseminate from a local focus of infection.

The study presented here aimed to obtain further information about the mechanism(s) used by *S. pyogenes* to survive within PMNs. For this purpose, transmission electron microscopy studies were performed on tissue isolated from mice infected with *S. pyogenes.* C3H/HeN female mice (Harlan-Winkelmann, Borchen, Germany) were intravenously inoculated with 10^5 CFU of *S. pyogenes* strain A20 (M type 23), a human isolate obtained from the German Culture Collection (DSM 2071). For infection, *S. pyogenes* was cultured at 37°C in Todd-Hewitt broth (Oxoid, Basingstoke, United Kingdom), supplemented with 1% yeast extract (THY). Bacteria were collected in mid-log phase, washed twice with sterile phosphate-buffered saline (PBS), and diluted to the required inoculum, and the number of viable bacteria was determined by counting CFU after the bacteria were diluted and plated in blood agar plates (GIBCO, Paisley, United Kingdom) containing 5% horse blood. At 48 h postinoculation, mice were sacrificed and the organs were fixed with a fixation solution containing 2% glutaraldehyde and 5% formaldehyde in a cacodylate buffer (0.1 M cacodylate, 0.09 M sucrose, 0.01 M $CaCl_2$, 0.01 M $MgCl_2$ [pH 6.9]) by perfusion through the tail vein. Dissected organs were further fixed by diffusion for 2 h on ice, washed with the cacodylate buffer, postfixed with 1% aqueous osmium tetroxide for 2 h at room temperature, dehydrated with a graded series of acetone, and embedded in the low-viscosity resin Spurr. After polymerization for 8 h at 70°C, ultrathin sections were cut, counterstained with uranyl acetate (5 min) and lead citrate (2 min), and examined in a Zeiss TEM910 transmission electron microscope at an acceleration voltage of 80 kV and at calibrated magnifications. The electron photograph displayed in Fig. 1A shows that streptococcus can be found within PMNs in infected tissue with intracellular bacteria located in large membrane-bound vacuoles. The ability of *S. pyogenes* to breach the phagocytic vacuole and escape into the cytoplasm of the PMNs is illustrated in Fig. 1B. One microorganism already free in the cytoplasm of a PMN in the proximity of the phagocytic vacuole is also visible in Fig. 1B. The photograph in Fig. 1C shows that *S. pyogenes* not only remains viable in the cytoplasm of the PMN but also is able to divide. The mechanism by which the vacuolar membrane is breached by *S. pyogenes* in order to gain access into the cytoplasm has not yet been elucidated. In the case of *Listeria monocytogenes*, the cholesterol-dependent listeriolysin enables the microorganism to escape from a membrane-bound vacuole into the cytosol following invasion into the host cells (2). In this regard, *S. pyogenes* produces two cytolytic toxins, streptolysin S and streptolysin O. Their relevance as virulence factors has been demonstrated by the reduced virulence of streptolysin-deficient mutants in mouse models of infection (1, 8). Additional studies are under way to evaluate the contribution of these cytolytic toxins to bacterial escape from the phagocytic vacuole into the cytoplasm of PMNs.

Two types of colonies were observed after homogenates of tissue isolated from mice infected with *S. pyogenes* were plated (Fig. 2A). Most colonies were opaque and had wrinkled surfaces, similar to the characteristics of colonies that originated from broth-grown bacteria (Fig. 2B). However, some other colonies exhibited glossy surfaces and were highly encapsulated. In order to determine the host environment where the different phenotypes had arisen, spleen cells were isolated from mice infected with *S. pyogenes* at 48 h of infection and treated with 100 μg of gentamicin (Sigma, Munich, Germany) per ml for 1 h at 37°C. Gentamicin kills extracellularly located

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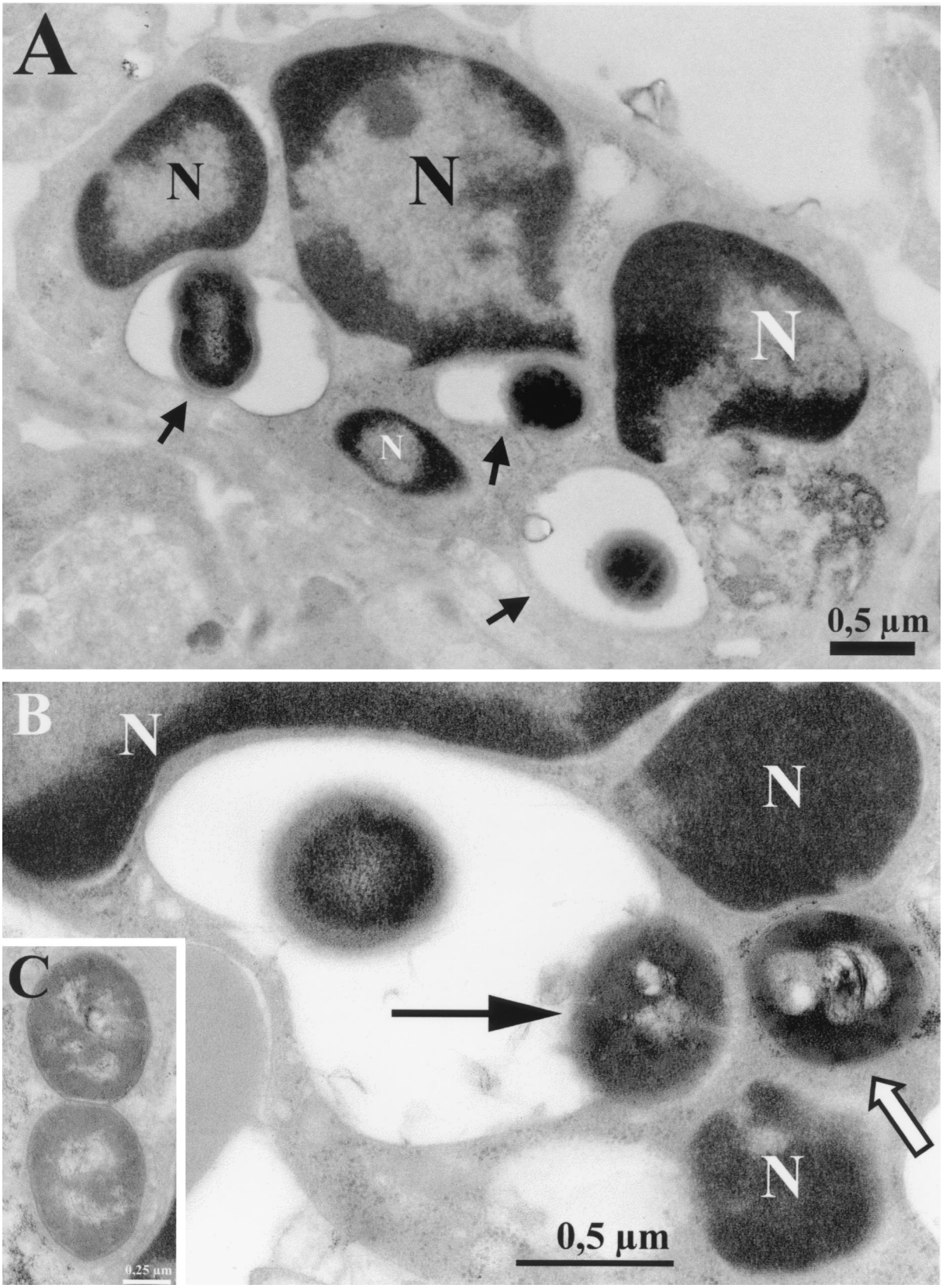

FIG. 1. Transmission electron photographs of *S. pyogenes*-infected PMNs present in the spleens of mice at 48 h postinfection. (A) *S. pyogenes* can be found in large vacuoles within phagocytic cells (filled arrow). (B) *S. pyogenes* organisms in the process of escaping from the phagocytic vacuole into the cytoplasm of the PMNs (filled arrow) and already free in the cytoplasm of the PMN in the proximity of a phagocytic vacuole (open arrow). (C) Dividing *S. pyogenes* in the cytoplasm of a PMN. The different lobules of the nuclei of PMNs are indicated by N.

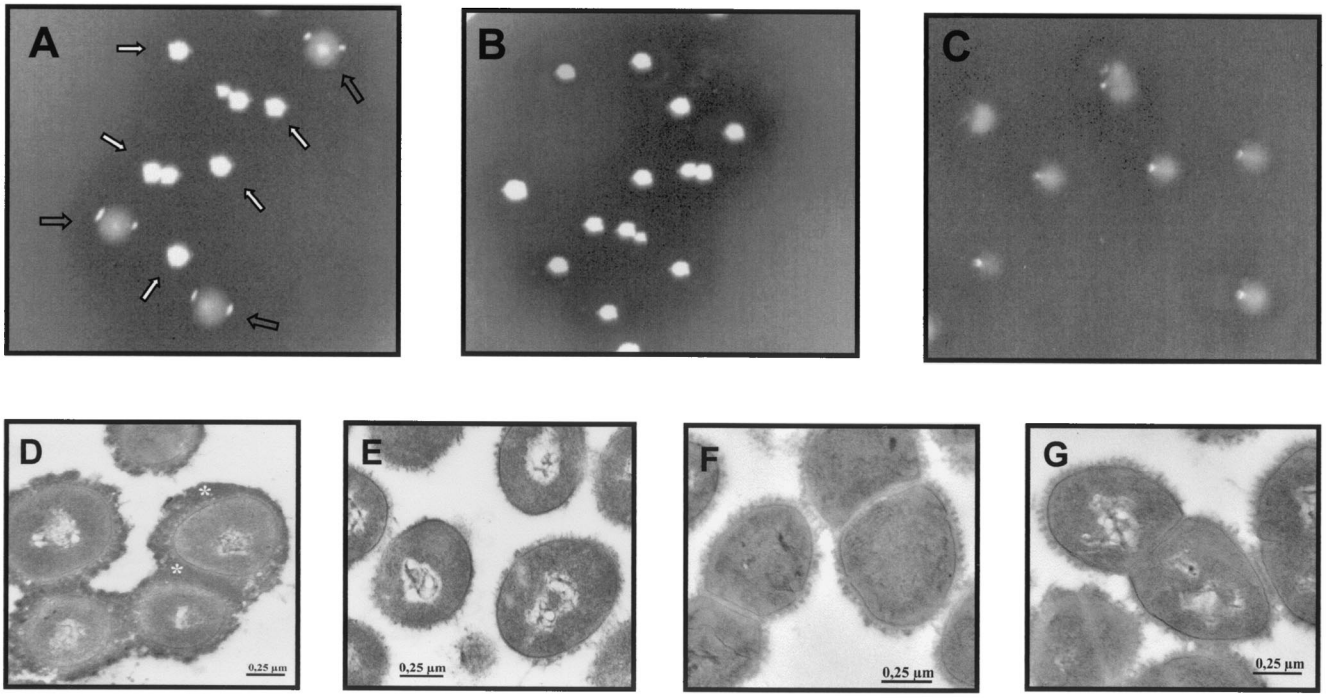

FIG. 2. Photographs showing the phenotypic switching of intracellularly located *S. pyogenes*. (A) Two types of colonies raised after spleen homogenates from *S. pyogenes*-infected mice were plated. Shown are opaque, wrinkled colonies (white arrows) similar to broth-grown colonies and highly capsulated, glossy colonies (gray arrows). (B) Colonies emerging from broth cultures exhibited the opaque, wrinkled phenotype. (C) Colonies generated from intracellularly located bacteria obtained after gentamicin treatment of PMNs isolated from *S. pyogenes*-infected mice exhibited the highly capsulated, glossy phenotype. (D and E) Transmission electron photographs of highly encapsulated *S. pyogenes* recovered from glossy colonies before (D) and after (E) treatment with hyaluronidase. The capsule is indicated by *. (F and G) Transmission electron photographs of *S. pyogenes* recovered from opaque, wrinkled colonies before (F) and after (G) treatment with hyaluronidase.

S. pyogenes but is limited in its ability to gain access to intracellular organisms. After 1 h, cells were extensively washed to eliminate traces of gentamicin and plated in blood agar. All colonies emerging from these intracellularly located microorganisms exhibited the glossy phenotype (Fig. 2C). In order to demonstrate that the glossy phenotype was due to an overproduction of capsule, bacteria isolated from both glossy and opaque colonies were treated with hyaluronidase (Sigma) for 45 min at 37°C and examined by transmission electron microscopy. The electron photograph displayed in Fig. 2D shows the broad capsules present in microorganisms obtained from glossy colonies. The capsule is almost completely lost after treatment with hyaluronidase (Fig. 2E). Bacteria isolated from opaque, wrinkled colonies were poorly encapsulated (Fig. 2F), and their appearance was not significantly modified after treatment with hyaluronidase (Fig. 2G).

To exclude the possibility that bacteria surviving gentamicin treatment are the result of spontaneous gentamicin-resistant microorganisms arising during the assay, 10^5 CFU of *S. pyogenes* was either added to 5 ml of homogenized spleen tissue or to 10^6 PMNs at 4°C to prevent bacterial uptake. Gentamicin was immediately added, and the samples were incubated at 37°C for 1 h. After incubation, samples were appropriately diluted and plated. No gentamicin-resistant organisms were detected in these assays, indicating that *S. pyogenes* remained susceptible to this antibiotic. These results indicate that intracellularly located *S. pyogenes* organisms undergo phenotypic switching. The mucoid phenotype was also observed after gentamicin treatment of purified PMNs infected in vitro with *S. pyogenes* following a previously described protocol (10). Intracellularly located microorganisms were as susceptible to gentamicin as broth-grown bacteria, as was indicated by their inability to grow in vitro in the presence of this antibiotic.

The ability to undergo phenotypic switching allows the bacteria to exploit different niches within the host by responding to environmental signals through the regulation and expression of virulence factors. Phenotypic variations in streptococcal strains in response to biological selection pressure in human blood or in mouse passaging have been noted, and mucoid-phenotype-selected variants were clearly demonstrated to be more virulent when they were tested in a mouse model of infection (4, 12). Therefore, we next investigated whether *S. pyogenes* virulence was affected by the intracellular milieu in PMNs. For this purpose, BALB/c mice, which have previously been shown to be very resistant to infection by *S. pyogenes* (6, 9), were intravenously injected with either gentamicin-treated purified *S. pyogenes*-infected PMNs, with uninfected PMNs, or with an inoculum of broth-grown *S. pyogenes* containing a number of microorganisms similar to the number of infected PMNs. PMNs were purified from the spleens of infected (48 h of infection with 10^7 CFU of *S. pyogenes*) or uninfected mice by positive selection by using MiniMACS magnetic microbeads

according to the manufacturer's instructions (Miltenyi Biotec Inc., Bergisch-Gladbach, Germany). Briefly, spleen cells were labeled with biotin-conjugated anti-mouse Ly-6G antibodies (Pharmingen), mixed with magnetic beads coated with streptavidin (catalog no. 130-048-101; Miltenyi Biotec), and incubated at 4°C for 30 min. The $RB6^+$ population (PMNs) was positively selected in a MiniMACS separation unit (Miltenyi Biotec), washed in PBS, and injected intravenously into naïve mice. The purity of the cell population was monitored by incubation with fluorescein isothiocyanate-conjugated anti-biotin antibodies (Miltenyi Biotec) followed by FACScan analysis. Ninety-nine percent of the purified population was found to contain $RB6^+$ cells. The actual number of bacteria contained within PMNs was determined by performing serial dilutions and plating in blood agar. Mice were monitored for survival over a period of 14 days. At 96 h of infection, mice were sacrificed by CO_2 asphyxiation and bacteria in specific organs were enumerated by plating 10-fold serial dilutions of tissue homogenates on blood agar plates. Colonies were counted after 24 h of incubation at 37°C. Numbers of viable bacteria in the blood of infected mice were also determined by collecting blood samples from the tail vein at different times postinoculation and by plating serial dilutions in blood agar. Results shown in Fig. 3 indicate that intracellularly located bacteria were much more virulent than broth-grown bacteria. All mice inoculated with gentamicin-treated PMNs isolated from mice infected with *S. pyogenes* succumbed to infection between days 4 and 6 postinoculation, whereas 100% of mice that were either infected with bacteria grown in broth or injected with uninfected PMNs survived (Fig. 3A). The superior virulence of intracellularly located bacteria was also evidenced by the loads of bacteria in the reticuloendothelial organs of infected mice (Fig. 3B), which were much higher than the loads in the control group. Mice inoculated with PMNs purified from the spleens of uninfected donors did not develop infection or other side effects and survived the treatment.

We then tested whether intracellularly and extracellularly located streptococci isolated from infected tissue differed in their levels of virulence. For this purpose, mice were infected with *S. pyogenes* intravenously and the spleens were removed from infected mice at 48 h postinoculation. Single-cell suspension procedures were performed, and cell-free extracellular bacteria were separated from cell-associated intracellular bacteria by differential centrifugation at $115 \times g$ for 10 min. The pellet containing cell-associated microorganisms was treated with 100 μg of gentamicin per ml for 1 h at 37°C and washed with PBS to eliminate traces of gentamicin. To assess the virulence of *S. pyogenes* organisms contained in the different fractions, naïve mice were injected intravenously with cell-containing bacteria (intracellular) and either a similar number of cell-free bacteria (extracellular) or broth-grown bacteria. Bacteremia was determined in the different groups of infected mice at 72 h postinoculation. The results displayed in Fig. 3C show that both extracellular and intracellular *S. pyogenes* organisms were more virulent than broth-grown microorganisms, but still bacteria isolated from the intracellular environment were significantly (t test, $P < 0.05$) more virulent than those extracellularly located. Thus, phenotypic switching exhibited by *S. pyogenes* within PMNs not only resulted in altered cellular characteristics, such as morphology and capsular expression,

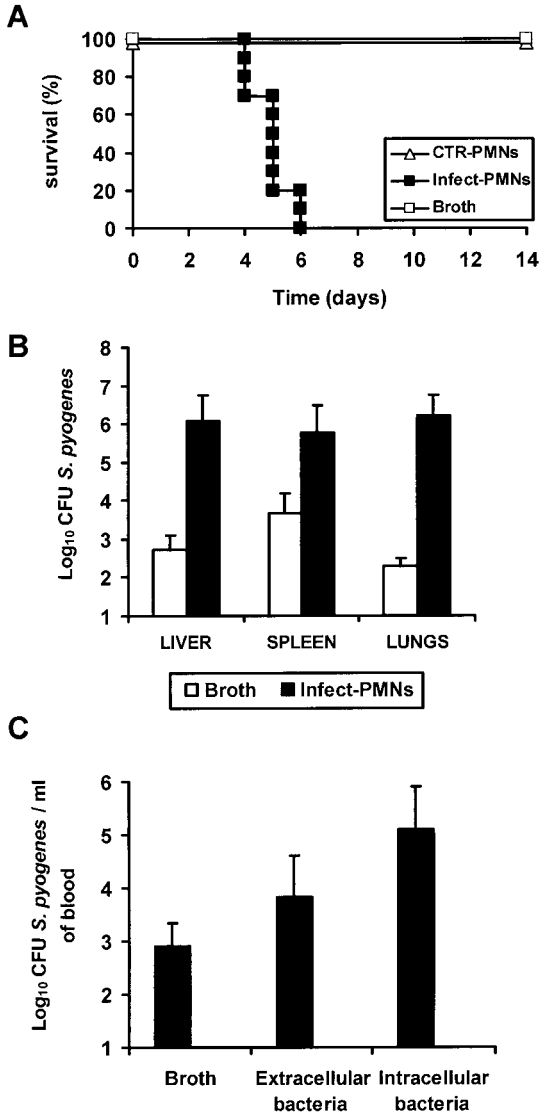

FIG. 3. Survival rates (A) and bacterial loads in the tissues (B) of mice intravenously inoculated with either gentamicin-treated spleen cells isolated from *S. pyogenes*-infected mice (■, filled bars) or broth-grown *S. pyogenes* (□, open bars). Mice inoculated with PMNs purified from the spleens of uninfected donors were used as the control (△). (C) Bacteremia in mice inoculated with similar numbers of either intracellularly or extracellularly located *S. pyogenes* organisms isolated from tissues of infected mice. Mice inoculated with a comparable number of broth-grown bacteria were used as controls. Bars represent the means ± standard errors of the means of results from experiments with five mice per group.

but also in increased virulence. Supporting this notion, Voyich et al. (13) have recently demonstrated that *S. pyogenes* differentially regulates a set of genes during phagocytic interaction with human PMNs which appear to be involved in bacterial immune evasion. In addition, genes controlled by the Ihk-Irr two-component gene-regulatory system of *S. pyogenes* were up-regulated during the phagocytosis of PMNs, indicating that these genes might be essential for the survival of *S. pyogenes* within phagocytic cells (13).

Therefore, the ability of *S. pyogene*s to sense and respond to

environmental signals within the host by regulating virulence functions required for immediate survival might constitute a key component to streptococcal pathogenesis.

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