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Iron Starvation Causes Release from the Group A Streptococcus of the ADP-Ribosylating Protein Called Plasmin Receptor or Surface Glyceraldehyde-3-Phosphate-Dehydrogenase

ZEHAVA EICHENBAUM, BRIAN D. GREEN, AND JUNE R. SCOTT*

Department of Microbiology and Immunology, Emory University, Atlanta, Georgia 30322

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In many pathogenic bacteria, iron starvation serves as an environmental signal that triggers the expression of virulence factors, many of which are found on the cell surface or secreted into the culture supernatant. Using the chelating agent nitrilotriacetic acid, we have established conditions for iron starvation of the important human pathogen *Streptococcus pyogenes* (the group A streptococcus) and determined that iron limitation results in the specific appearance of several new proteins in the culture supernatant. One of these supernatant proteins is the ADP-ribosylating protein known as streptococcal plasmin receptor (Plr) or as the streptococcal surface glyceraldehyde-3-phosphate-dehydrogenase because of its other activities. Upon iron starvation, Plr is specifically released into the culture supernatant in a time-dependent manner, and its appearance in the supernatant is not accompanied by induction of *plr* mRNA synthesis. Release of Plr from the bacteria may be important for the virulence of group A streptococci and the manifestation of diseases.

Streptococcus pyogenes (group A streptococcus [GAS]), a common pathogen of the human pharynx and skin, usually causes mild and self-limiting infections such as pharyngitis and impetigo. In some cases, however, these diseases can lead to serious immunological complications like rheumatic fever, acute glomerulonephritis, and rheumatic heart disease. In addition, GAS causes several severe and life-threatening invasive diseases, including myositis, necrotizing fasciitis, and the newly described streptococcal toxic shock syndrome (STSS) (4, 10). STSS is a rapidly progressive, destructive soft-tissue infection that is characterized by shock, bacteremia, and multiorgan failure. There is a high mortality (30%) associated with STSS (39).

In recent years, a significant increase in the number of serious and aggressive GAS infections has been observed worldwide. An increasing incidence of severe deep-tissue invasive diseases and acute rheumatic fever has been reported in the United States, Europe, and Australia (17, 38, 40, 44). In contrast to previous severe GAS infections, in which poor socioeconomic condition, poor hygiene, and substandard medical care were considered to be major contributing risk factors, infections in recent years have been shown to affect people of all ages and do not appear to be associated with the risk factors previously considered to be important (38).

With the exception of certain lactococci (2), iron is required by virtually all microorganisms to support vital cellular processes. It is required for the functioning of cytochromes in aerobic bacteria and serves as a cofactor for many enzymes, including ribonucleotide reductase, nitrogenase, peroxidase, catalase, and succinic dehydrogenase. In the human host the concentration of free iron that is available to infecting bacteria is very low (7, 22). Many pathogenic bacteria use this low free-iron concentration as a signal to regulate expression of virulence factors (22, 26, 29). These include Shiga toxin from *Shigella dysenteriae* (41), Shiga-like toxins from enterohemorrhagic *Escherichia coli* (9), diphtheria toxin from *Corynebacterium diphtheriae* (5), and exotoxin A from *Pseudomonas aeruginosa* (45). To gain a better understanding of the interaction between GAS and the human host and to begin a search for new GAS virulence factors, we have initiated studies to determine the effect of iron starvation on GAS growth and the associated change in protein profiles from corresponding culture supernatants.

* Corresponding author. Phone: (404) 727-0402. Fax: (404) 727-3659. Electronic mail address: scott@microbio.emory.edu.

MATERIALS AND METHODS

Bacterial strains. GAS strain JRS4 is a streptomycin-resistant derivative of type M6 strain D471 (37). The GAS strain JRS301 is a type M1 strain that was isolated from a patient diagnosed with STSS (35).

Media. Cells were grown in Todd-Hewitt broth containing 5% yeast extract and 0.1 M Tris HCl (ZTHYB) that was adjusted to pH 6.9 prior to autoclaving. Following sterilization, ZTHYB reached a pH of 7.3. ZTHYB medium was depleted of iron by adding 18 mM nitrilotriacetic acid trisodium salt (NTA) and a mixture (defined as MBC) of $MgCl_2$, $CaCl_2$, $MnCl_2$, and $ZnCl_2$ at 1 mM each. Iron was restored to the medium by addition of 13 mM ferric citrate or 13 mM ferrous sulfate. All glassware was washed, soaked for 2 h in chromic-sulfuric acid (sulfuric acid and Chromerge [Fisher]), and rinsed with double-distilled water.

Preparation of culture supernatant proteins. Culture samples were centrifuged at 6,000 × *g* for 20 min and the supernatants were filtered through 0.45-μm-pore-size filters and concentrated 30-fold using centrifugal concentrators (Amicon) with a 10,000-molecular-weight cutoff (Centricon-10). The concentrated supernatants were stored at 20°C.

Total cell extracts. GAS cells were lysed with mutanolysin as described previously (28), except that following cell lysis, the sample was centrifuged (for 5 min) and the supernatant was defined as the total soluble cell extract. The pellet containing membranes and cell debris was subsequently resuspended in water and was labeled as the insoluble cell extract.

SDS-PAGE and Western analysis. Supernatant protein samples were standardized with respect to the number of cells in the corresponding culture, separated by sodium dodecyl sulfate–12.5% polyacrylamide gel electrophoresis (SDS-PAGE), and stained with Coomassie brilliant blue (20). For immunological analysis, proteins were transferred electrophoretically to a nitrocellulose membrane (8) and reacted with mouse anti-Plr polyclonal antibody received from R. Lottenberg. Antibody-antigen complexes were visualized as described previously (36).

RNA slot blot analysis. Total RNA was extracted from GAS cultures as described by McDonald et al. (27), except that cells were grown in ZTHYB and glycine was omitted from the medium. RNA was pelleted by sedimentation through 5.7 M CsCl (15) and transferred to a Zeta-probe membrane (Bio-Rad) as previously described (43), using a Bio-Rad microfiltration apparatus. The membrane was reacted with probes generated by PCR amplification and labeled with [^{32}P]dATP by random priming. The sequences of the primers used were as follows: *plr*-specific right primer, ATCGTGAAGAGCTTTAGCC; *plr*-specific left primer, CGTCTTGCATTCCGCCG; *recA*-specific right primer, CTGATG CTACTGCCATAGCAG; *recA*-specific left primer, GCGTTCAGGAAGTCT

AGCTC; *ska*-specific right primer, GGTGTCCCTGTATAACGCA; *ska*-specific left primer, CGGGATCCTGGGATGTTTGCACTGCTG.

RESULTS

GAS growth under conditions of iron depletion. GAS strains grow rapidly in Todd-Hewitt broth containing 5% yeast extract (THYB). To remove iron from this complex medium, we tested several different chelators at various concentrations, including Chelex 100, desferal mesylate, 2,2-dipyridyl, ethylenediamine-di-*o*-hydroxy phenylacetic acid (EDDA), and nitrilotriacetic acid (NTA). Starvation was defined as a decrease in growth rate in response to the addition of chelator during the early log phase of growth. Because none of these chelators is completely specific for iron, we required that the chelation effect be reversible by the addition of ferrous sulfate or ferric citrate concurrently with the addition of chelator. In addition, we wished to use conditions that did not kill the cells. Therefore, we also required that ferrous sulfate or ferric citrate be able to restore growth when added several hours after the addition of chelator.

After extensive preliminary experimentation, we chose the iron chelator NTA. Since the addition of NTA resulted in an increase in the pH of THYB, 0.1 M Tris (pH 7.3) was added to buffer the medium (designated ZTHYB). To prevent any effects by chelation of bivalent cations in addition to iron, a mixture of manganese chloride, zinc chloride, magnesium chloride, and calcium chloride (MBC) was added to both the experimental and control cultures at the time of addition of the chelator.

During the early exponential phase of growth on ZTHYB, the culture was treated with 18 mM NTA or with NTA and 13 mM ferric citrate (or 13 mM ferrous sulfate). Addition of NTA resulted in a pronounced decrease in the growth rate as measured by optical density. The doubling time of the culture increased from 65 min to 110 min in the presence of NTA (Fig. 1). This decrease in growth rate was prevented by addition of ferric citrate or ferrous sulfate. The iron-starved cells also entered the stationary growth phase at a lower cell density as compared with the control culture. When ferric citrate or ferrous sulfate was added to the cells in addition to NTA, the growth of the culture was identical to that of the control (untreated culture grown on ZTHYB) (data not shown). This indicates that the effect on growth of NTA results from lack of iron and demonstrates the requirement for iron by this strain of GAS.

Even 20 h after NTA addition, there was no massive cell death; only a small (15%) decrease in the optical density of the culture occurred. Furthermore, a comparison of the total cell count (determined microscopically) to the viable count at several points during growth showed no detectable difference. Therefore, the majority of the iron-starved cells remain viable. This demonstrates that the growth inhibition, induced by iron depletion, is completely reversible by plating on iron-containing medium.

Iron depletion induces changes in the profile of supernatant proteins. To begin an analysis of new proteins produced by GAS in response to iron starvation, we examined the protein profile of culture supernatants by SDS-PAGE analysis. Several proteins were found in the iron-depleted culture (Fig. 2, lanes 4, 6, and 8) that were absent from the culture grown in the presence of iron (Fig. 2, lanes 3, 5, and 7). We focused our initial efforts on a major protein band that migrates with an apparent molecular mass of about 42 kDa. This protein appeared only under iron starvation conditions and was present in the supernatants from both the M6 GAS strain JRS4 (Fig. 2) and the M1 strain JRS301 (data not shown). In addition, the appearance of this protein in the culture supernatant was time dependent. It was present at low levels in the supernatant 2 h following addition of NTA (Fig. 2, lane 2) and gradually increased over time (Fig. 2, lane 4, 6, and 8).

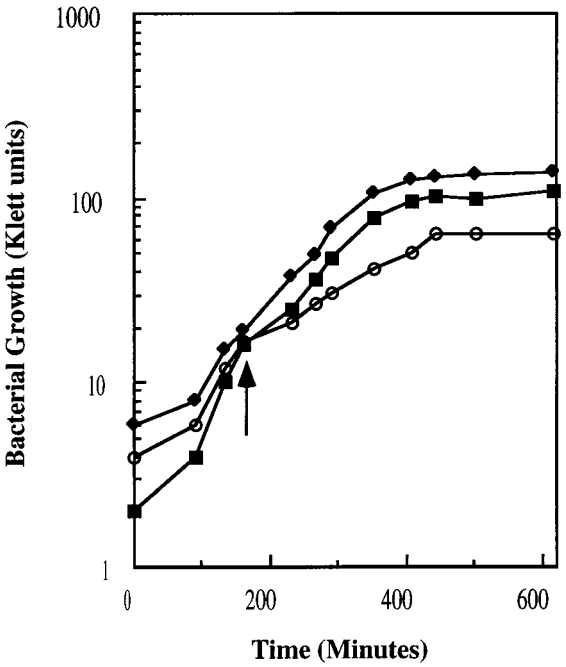

FIG. 1. Growth of GAS under iron-depleted conditions. Cells were grown in ZTHYB in tightly capped flasks without agitation. At early exponential phase of growth, cultures were treated with either 18 mM NTA and MBC (○) or NTA, MBC, and either 13 mM ferric citrate (■), or 13 mM ferrous sulfate (◆). The arrow indicates time of NTA addition.

The 42-kDa protein is Plr. The 42-kDa protein band from the culture supernatant of cells grown under iron starvation conditions was transferred to a polyvinylidene difluoride membrane, and the N-terminal sequence of the protein was determined. The first 21 amino acids revealed 100% homology to the streptococcal plasmin receptor (Plr) (24), which is also known as the streptococcal surface glyceraldehyde-3-phosphate-dehydrogenase (SDH) (32). Previous reports indicated

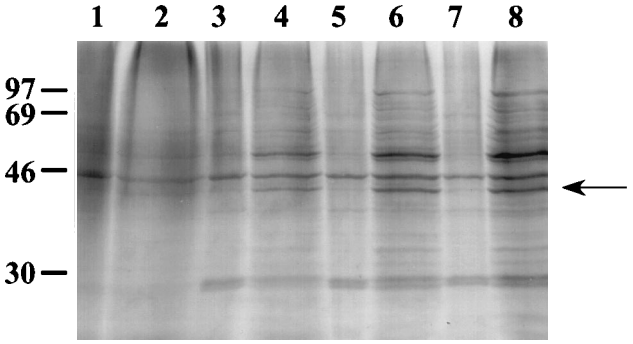

FIG. 2. Protein profile obtained by SDS-PAGE of culture supernatant during iron starvation. Proteins were prepared from culture supernatant samples taken 2, 4, 7, and 20 h following addition of 18 mM NTA and MBC (lanes 2, 4, 6, and 8, respectively) or addition of NTA, MBC, and 13 mM ferric citrate (lanes 1, 3, 5, and 7, respectively). The molecular masses (in kilodaltons) of standard proteins (Rainbow marker; Amersham) are indicated on the left. The arrow points to the 42-kDa protein that was identified as Plr.

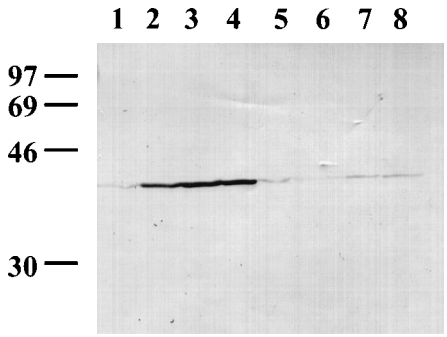

FIG. 3. Western blot of supernatant proteins reacted with mouse anti-Plr. Proteins were prepared from culture supernatant samples taken at 2, 4, 7, and 20 h following addition of 18 mM NTA and MBC (lanes 1, 2, 3, and 4, respectively) or addition of NTA, MBC, and 13 mM ferric citrate (lanes 5, 6, 7, and 8, respectively). The molecular masses (in kilodaltons) of standard proteins (Rainbow marker; Amersham) are indicated on the left.

that this protein is located on the streptococcal cell wall (6, 32). This is consistent with its absence from the supernatants of cultures grown under iron-replete conditions (Fig. 2, lanes 1, 3, 5, and 7).

The identity of the 42-kDa protein as Plr was further confirmed by Western blot (immunoblot) analysis using a polyclonal antibody directed against Plr (Fig. 3). A very low concentration of Plr was detected in the supernatant of the control culture at all times, while the amount of Plr in the supernatant of the iron-depleted culture increased significantly after starvation.

Iron depletion does not increase the amount of *plr* message. To determine whether the presence of Plr in the supernatant of iron-starved GAS cultures is due to induction of transcription of *plr* or to specific release of Plr from the cell wall, the amount of *plr* mRNA was determined. To ensure accurate quantitation, *recA* mRNA and streptokinase (*ska*) mRNA were used as internal controls. The mRNA was prepared from JRS4 at the time of addition of NTA (0 min) and 120 and 240 min after the addition of NTA. A significant increase in the level of Plr protein in the supernatant of iron-starved cultures had been seen at these times (Fig. 3). Dilutions of the RNA samples were used for hybridization in slot blots probed with each of the three different PCR-amplified DNA fragments, *plr*, *recA*, and *ska* (Fig. 4). The hybridization with *recA* (Fig. 4B) and *ska* (Fig. 4C) showed that the amount of mRNA in the extracts prepared at the different times after NTA addition was constant. During the 4 h of growth with NTA, no significant change in the *plr* mRNA level was detectable (Fig. 4A), indicating that iron depletion does not induce transcription of this gene. This suggests that starvation for iron either causes specific release of Plr from the surface or causes lysis of the GAS culture.

Iron depletion does not induce cell lysis. It seemed unlikely that iron starvation induces cell lysis, because even after overnight starvation for iron, only a very small decrease (15%) in the optical density of the culture was seen. Nevertheless, to address the possibility of cell lysis, we compared the protein profiles of both the soluble and the insoluble fractions of the total cell extracts (Fig. 5, lanes 2 and 3, respectively) with those of the supernatants from iron-replete (Fig. 5, lanes 4, 5, 7, and 8) and iron-depleted (Fig. 5, lanes 6 and 9) cells. Comparison of the protein profiles showed that there are fewer proteins in the supernatant of iron-starved cells than in the total cell extract. This indicates that the increase in the concentration of

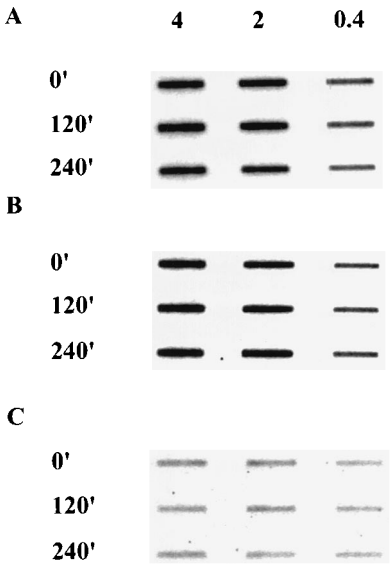

FIG. 4. Slot blot analysis of RNA from iron-starved culture. RNA was prepared from iron-starved cells at 0, 120, and 240 min following addition of NTA and MBC. Four, 2, and 0.4 μg of RNA per slot (as indicated at the top) were loaded for each time point. The membrane was reacted with probes specific to *plr* (A), *recA* (B), and *ska* (C).

Plr in the iron-starved culture supernatant is not due to an increase in overall lysis of the cells.

DISCUSSION

Relatively little is known about the role that iron plays in the physiology and virulence of streptococci, many of which are important human pathogens. There have been contradictory reports regarding a possible iron requirement for *Streptococcus mutans* (1, 3, 25), and iron was reported to be an essential nutrient for the growth of *Streptococcus pneumoniae* (42). In GAS, iron can stimulate cell growth and the production of streptolysin S (16). In the experiments described in this report we found evidence that iron is essential for the growth of GAS. When the iron chelator NTA was added to log-phase cultures

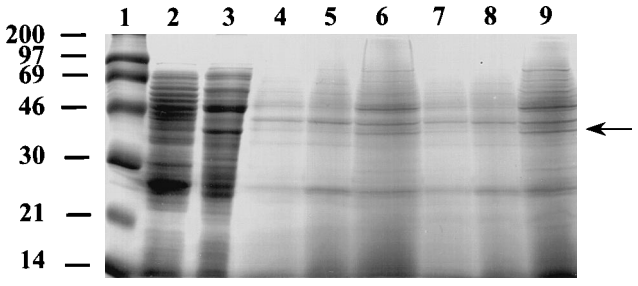

FIG. 5. Protein profiles obtained by SDS-PAGE of total cell extracts and culture supernatants. Proteins were prepared from whole cells (lanes 2 and 3) and culture supernatants (lanes 4 to 9). Cells harvested from culture samples taken 4 h following addition of 18 mM NTA and MBC were washed and lysed. The soluble fraction of the cell lysate is in lane 2, the insoluble fraction is in lane 3. Proteins were prepared from culture supernatants taken 4 and 7 h (lanes 4 to 6 and 7 to 9, respectively) after addition of the following: 18 mM NTA, MBC, and 13 mM ferrous sulfate (lanes 4 and 7); NTA, MBC, and 13 mM ferric citrate (lanes 5 and 8); NTA and MBC (lanes 6 and 9). Lane 1 contains a standard protein marker (Rainbow marker; Amersham); molecular masses (in kilodaltons) are indicated on the left. The arrow points to the 42-kDa protein that was identified as Plr.

of *S. pyogenes* JRS4, there was an immediate reduction in growth rate.

Iron plays a major role in the virulence of many bacterial pathogens (22, 26). In addition to regulating genes needed for iron acquisition, iron concentration often regulates virulence genes that are not directly involved in its uptake (22, 26). Many of these genes encode proteins that are either associated with the cell surface or secreted into the culture medium. When the GAS strains JRS4 and D471 were grown under iron-depleted conditions, we found several new proteins in the culture supernatants. One of the most prominent of these was identified as Plr, also known as SDH (24, 32).

Although the Plr protein has been reported to be present on the outer surface of GAS under normal growth conditions (6, 32), it lacks a conventional signal sequence for processing by the Sec pathway (24). In agreement with this, we found that the N-terminal amino acid sequence of the Plr protein in the GAS culture supernatant after iron starvation corresponds to that predicted from the DNA sequence. It is possible that Plr is secreted by a specific pathway similar to the type III secretion pathway involved in export of glyceraldehyde-3-phosphate-dehydrogenase in enteropathogenic *E. coli* (11, 18). No such pathway has yet been identified in gram-positive bacteria, but the *Listeria monocytogenes* virulence factor internalin, which also lacks a signal sequence, is found on the bacterial cell surface as well (13).

Although in other bacterial pathogens, low iron concentration usually induces transcription of new proteins, including virulence factors (22, 29), in GAS *plr* transcription is not induced by iron starvation. However, since the mechanism of release of Plr from the cell surface is not known, it is possible that iron depletion induces transcription of other proteins involved in this process. When GAS are grown under iron-rich conditions, Plr appears to be tightly associated with the cell wall (32), although it lacks the cell wall-spanning and membrane-anchoring motifs that have been identified for surface proteins of gram-positive bacteria (12, 31). When GAS is starved for iron, no cell lysis is induced but Plr is released from the cell. An enzymatic activity has been found on the surface of *S. mutans*, and also of GAS, which causes the release of multiple surface polypeptides whose size is not altered during release (21). It is possible that this or a similar activity is involved in release of Plr from the GAS surface, since the released protein is the same size as the intracellular form. However, Plr release is dependent on iron depletion conditions, and this was not investigated for the *S. mutans* surface protein-releasing enzyme activity.

The release of Plr from the GAS surface under conditions of low iron concentration, similar to those found in the human host, may have importance for the virulence of the organism and the type of disease syndromes it causes. If the Plr remained bound to the bacterial surface, it could act only at the host sites with which the streptococcus was associated. Instead, when the Plr is released, it becomes freely diffusible and each molecule can act on a different host target cell. This ability to diffuse should therefore amplify the effect of Plr.

There are several possible roles for secretion of Plr in infections by GAS, based on the known properties of this protein. This protein was named for its ability to bind plasmin, the active protease form of plasminogen. Binding of plasmin to Plr prevents its inactivation by α_2-antiplasmin in some situations (23). It is therefore possible that Plr in combination with plasmin may associate with host tissues at sites that are not adjacent to those at which the streptococci reside. These plasmin-Plr complexes might lead to tissue destruction by the proteolytic activity of the plasmin, and this might facilitate invasion of deeper tissue sites by the bacteria.

Plr protein (also called SDH) has two additional different enzymatic activities: it acts as a glyceraldehyde 3-phosphate-dehydrogenase and as an ADP-ribosylating enzyme (32, 33). Plr mediates NAD-dependent autoribosylation and the transfer of ADP-ribose to free cysteine (33). Mono-ADP-ribosylation is a common mechanism by which eukaryotic cells modify protein structure and function. Many bacterial toxins, including diphtheria toxin (34), pseudomonas exotoxin A (34), pertussis toxin (14, 19), and cholera toxin (30), act by ADP-ribosylation of host proteins. It is possible that released Plr may also ADP-ribosylate human proteins during infection. Although GAS are extracellular pathogens, the release of the Plr from the streptococcal cell surface would allow it to enter host cells, where it might act on intracellular proteins. This Plr activity may be cytotoxic, like those of the above toxins, and lead to cell death. Alternatively, it may play a role in a signal transduction process to stimulate specific cellular responses.

Recently, a glyceraldehyde 3-phosphate-dehydrogenase (GA PDH) protein of enteropathogenic *E. coli* (EPEC) was reported to be secreted from EPEC and to play a role in bacterium-host communication (11). Contact with EPEC stimulates human epithelial cells in vitro by induction of inositol triphosphate and Ca^{2+} fluxes and protein phosphorylation. This leads to rearrangement of the host cytoskeletal components and facilitates the close approximation of the bacteria to the host cell surface (11). The GAPDH-related signal-transducing proteins that are secreted by EPEC strains in response to the presence of epithelial cells in vitro, like GAS Plr, lack a signal sequence. Although the stimulus for release of these EPEC proteins was not characterized in detail, secretion occurred in the absence of epithelial cells in tissue culture medium, but not in LB broth or M9 minimal medium (11). The free-iron concentration in tissue culture medium is likely to be quite low. Therefore, it is possible that, like the secretion of Plr by GAS, the signal that triggers the secretion of the EPEC protein may be low iron concentration. Further work is required to determine whether this idea is correct and to determine the significance of Plr release by GAS in infection.

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