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Temperature Regulation of the Streptococcal Pyrogenic Exotoxin A-Encoding Gene (*speA*)

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The gene encoding the bacterial superantigen streptococcal pyrogenic exotoxin A is often found in streptococcal strains associated with the recently described streptococcal toxic shock syndrome. Here we demonstrate that this gene is expressed at approximate fourfold higher levels in cells grown at 37°C when compared to cells grown at 26°C. This suggests there is increased production of this toxin when *Streptococcus pyogenes* is found in infections of the soft tissues and bloodstream, as opposed to *S. pyogenes* that have not breached the epithelial layers and are living on the surface of the skin.

The bacterial pathogen *Streptococcus pyogenes* can cause infection at a number of sites in humans, the most common being the pharynx (1). *S. pyogenes* can also cause skin and soft-tissue infections, including pyoderma, erysipelas, cellulitis, and necrotizing fasciitis (2). In recent years, severe invasive *S. pyogenes* infections associated with shock and organ failure have been reported with increasing frequency, and these infections have been termed streptococcal toxic shock syndrome (STSS) (6, 12, 13, 17).

Streptococcal pyrogenic exotoxins are suspected of playing a critical role in the pathogenesis of STSS (2, 5). There are at least three distinct streptococcal pyrogenic exotoxins, SpeA, SpeB, and SpeC (16), and in the United States most of the isolates from STSS produce SpeA (5). SpeA (molecular weight, 25,787) is a bacteriophage-encoded protein produced by *S. pyogenes* lysogens (16). When associated with human infections, SpeA acts as a bacterial superantigen that can clonally activate and expand T cells through interaction with the T-cell receptor (16). This interaction is dependent upon presentation by the major histocompatibility complex class II molecules. Superantigenic activity results in the overproduction of tumor necrosis factor alpha, interleukin-1β, interleukin-6, and other cytokines, and the overexpression of these proteins is a plausible mechanism to explain the shock and organ failure associated with STSS.

Many bacterial genes that encode virulence determinants are regulated in response to environmental signals (9, 11). Understanding the environmental conditions that lead to expression of virulence-associated genes is useful for determining when and where during infection the particular determinant is expressed. Because of the importance of SpeA in the pathogenesis of STSS, we began studies to elucidate if there are environmental signals that trigger SpeA production.

Competitive ELISA. In order to assay for SpeA expression a competitive enzyme-linked immunosorbent assay (ELISA) was developed (Fig. 1). Polyclonal serum against commercially purchased SpeA (Toxin Technologies) was raised by immunizing New Zealand White rabbits as described previously (8). The immunoglobulin (Ig) fraction of this polyclonal serum was isolated by using a protein G affinity column (Pharmacia) according to the manufacturer's recommendations. Recombinant SpeA (rSpeA) was obtained as described previously (8) and biotinylated by using NHS-LC-Biotin II (Pierce). A 5- to 10-fold molar excess of NHS-LC-Biotin II was added to 1 mg of rSpeA in 1 ml of phosphate-buffered saline. After 2 h on ice, free biotin was removed by gel filtration. Then, 96-well polystyrene flat-bottomed plates were coated overnight at 4°C with 1.5 μg of goat anti-rabbit IgG in 0.05 M bicarbonate (pH 9.8). Unlabeled rSpeA- or SpeA-containing samples were preincubated with labeled rSpeA (2 ng per well) and anti-SpeA antibodies. After 30 min at 37°C, 150 μl of 0.1 μg/ml streptavidin-alkaline phosphatase solution (Pierce) was added to the wells. An ELISA amplification system (Gibco/BRL) was added after 30 min at 37°C and the optical density (OD) at 492 nm (OD_{492}) was read. All samples were done in triplicate. As shown in Fig. 1, the reaction was linear for rSpeA concentrations of 0 to 100 ng.

Expression is not affected by external pH, osmolarity, or atmospheric conditions. The SpeA-producing *S. pyogenes* strain Ros (generous gift of D. L. Stevens, Idaho VA Medical Center) was grown in Todd-Hewitt medium (Difco) supplemented with 0.2% yeast extract (THY) in stationary flasks. Initially this medium was modified to vary in either osmolarity or pH. Osmolarity of the medium was raised by adding NaCl such that the final salt concentration was increased by either 0.2 or 0.4 M NaCl. The initial pH of the media was modified so that it was either 6.5, 7.0, 7.5 or 8.0. These media were buffered with either 0.25 M PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] or 0.25 M MOPS (morpholinepropanesulfonic acid) depending on the initial pH. There were no changes in expression of SpeA when either osmolarity or pH was altered (data not shown).

S. pyogenes Ros was grown under various atmospheric concentrations of CO_2 and O_2, as described by Caparon (3). The atmospheric concentrations of CO_2 and O_2 did not affect expression of this protein (data not shown).

Expression varies with growth temperature. When *S. pyogenes* Ros was grown in stationary cultures of THY at various temperatures, there was a difference in production of SpeA (Fig. 2A). This difference was evident throughout the growth cycle. At stationary growth phase, there was an approximate fourfold greater level of SpeA found in the cultures grown at 37°C when compared to those grown at 26°C. Other clinical isolates of *S. pyogenes* were tested for SpeA expression at the two temperatures, and all showed from 3.5 to 4-fold higher expression at 37°C than at 26°C (data not shown). These strains include *S. pyogenes* NY5 and three *S. pyogenes* clinical

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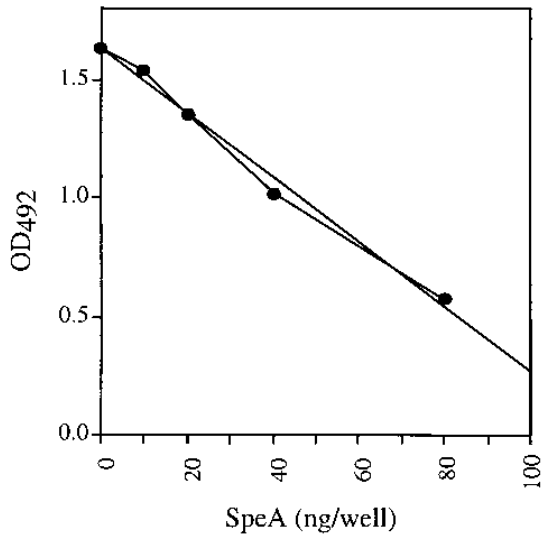

FIG. 1. Competitive ELISA for detection of SpeA. Assay was performed as described in the text. Solid line was determined by linear regression analysis.

isolates: strains JMH, Dav, and 1086 (from the strain collection of A. L. Bisno, VA Medical Center, Miami, Fla.).

Growth rate at the two temperatures differs. The growth rate of the organism differs at the two temperatures, and *S. pyogenes* Ros grows faster at 37°C than at 26°C (Fig. 2B). Thus the difference in expression cannot be solely attributed to temperature. *S. pyogenes* Ros was grown at 37 and 26°C and at various OD measurements the viable cell number was determined by plating out serial dilutions onto THY solidified with 1.5% agar. The number of viable cells per OD unit remained the same at the two temperatures (data not shown). Therefore, the difference in expression was not an artifact that resulted from having more cells, and thus more toxin, per OD unit at the higher temperature.

Expression is regulated at the level of transcription. Northern (RNA) blot analysis was performed to determine if the difference in the expression was reflected at the level of transcription. RNA was isolated from *S. pyogenes* by using the following protocol. Cells were grown in 100 ml of THY at the appropriate temperature to a low density, and glycine was added to 20 mM. Cells were harvested at mid-logarithmic phase by pouring them over 50 ml of frozen 100 mM Tris-HCl (pH 6.8)–2 mM EDTA–0.06% Na azide and centrifuged at 10,000 rpm (15 min, 4°C). The pellet was resuspended in 10.8 ml of 100 mM Tris-HCl (pH 6.8)–2 mM EDTA–2 mg of lysozyme per ml. After 20 min on ice, 1.2 ml of lysis buffer (0.5 M Tris-HCl [pH 6.8], 20 mM EDTA, 10% Sarcosyl) was added, and the tube placed in a boiling water bath for 5 min. Then, 120 μl of 2 M KCl was added, and the tube was left on ice for 30 min. The supernatant was collected and mixed with 1.6 ml of 0.5 M EDTA (pH 8.0), 200 μl of β-mercaptoethanol, and 5.4 g of CsCl. The solution was layered on 1.2 ml of CsCl solution (5.7 M CsCl in 0.1 M EDTA) in polyallomer tubes and centrifuged at 35,000 rpm for 12 h (20°C). The pellet was resuspended in 0.5 ml 10 mM Tris-HCl (pH 6.8), 5 mM EDTA, 2 mM Ribonucleoside-Vanadyl complex (Biolabs, Beverly, Mass.) and extracted once with chloroform:butanol (4:1). The RNA was precipitated with 2 volumes of ethanol, collected by centrifugation, and redissolved in 2 mM vanadyl-ribonucleosides in diethyl pyrocarbonate (DEPC)-treated water, and the RNA concentration was determined by measuring the OD_{260}.

Total cellular RNA was isolated from *S. pyogenes* Ros (*emm3* positive) grown at either 26 or 37°C and from *S. pyogenes* Ste grown at 37°C. *S. pyogenes* Ste is negative for both *speA* and the M protein encoding gene *emm*. Equal amounts of RNA, as determined by absorption at OD_{260}, were loaded per lane of an agarose-formaldehyde gel, fractionated by electrophoresis, blotted to nitrocellulose membranes, and hybridized with a *speA*-specific DNA probe (Fig. 3A, top). After exposure

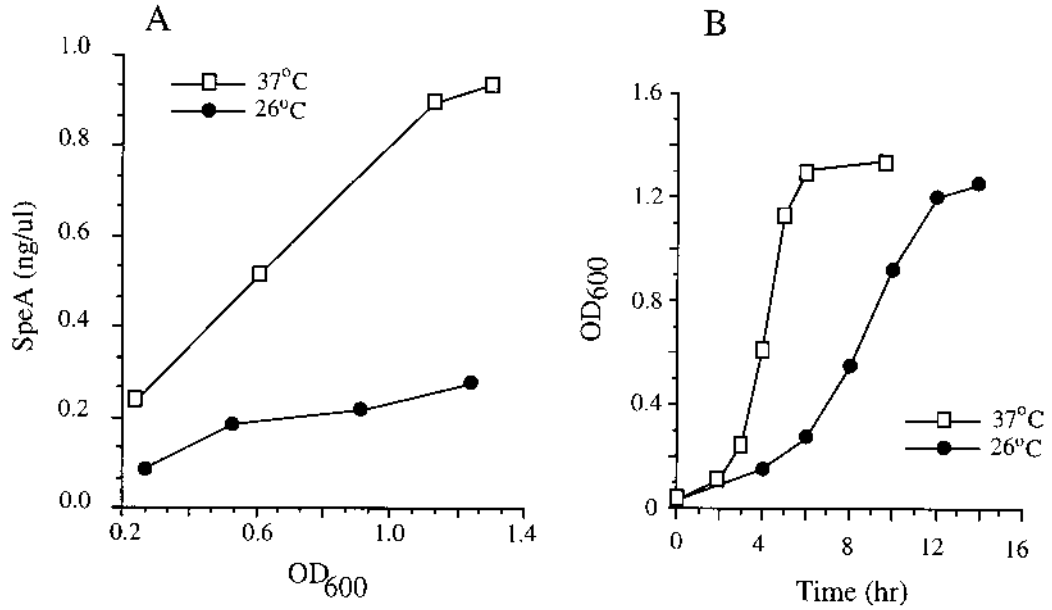

FIG. 2. Effect of temperature on the production of SpeA and growth rate of *S. pyogenes*. An overnight culture of *S. pyogenes* Ros was subcultured (1:100) into fresh THY and grown to stationary phase at either 37 or 26°C. Aliquots were taken at various points in the growth curve (indicated by OD_{600} readings), and SpeA was quantified by competitive ELISA. (A) Production of SpeA at 37 or 26°C. (B) Growth of *S. pyogenes* at 37 or 26°C.

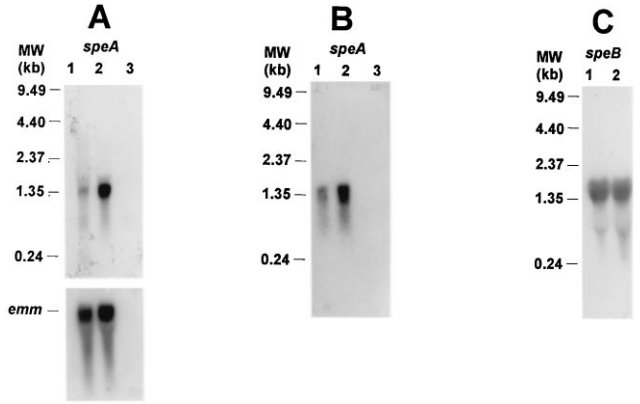

FIG. 3. Temperature regulation of *speA* is at the level of transcription. Total RNA was isolated from the indicated *S. pyogenes* strain grown at either 37 or 26°C and quantified by A_{260}, and equal amounts were fractionated by formaldehyde gel electrophoresis. (A) Lane 1, strain Ros (*speA*$^+$) grown at 26°C; lane 2, strain Ros grown at 37°C; lane 3, strain Ste (*speA* negative, *emm* negative) grown at 37°C. Top, hybridization with a *speA*-specific probe; bottom, same blot hybridized with an *emm*-specific probe. (B) Lane 1, strain NY5 (*speA*$^+$) grown at 26°C; lane 2, strain NY5 grown at 37°C; lane 3, strain Ste (*speA* negative, *emm* negative) grown at 37°C. Hybridization with a *speA*-specific probe. (C) Lane 1, strain Ros (*speB*$^+$) grown at 26°C; lane 2, strain Ros grown at 37°C. Hybridization with a *speB*-specific probe.

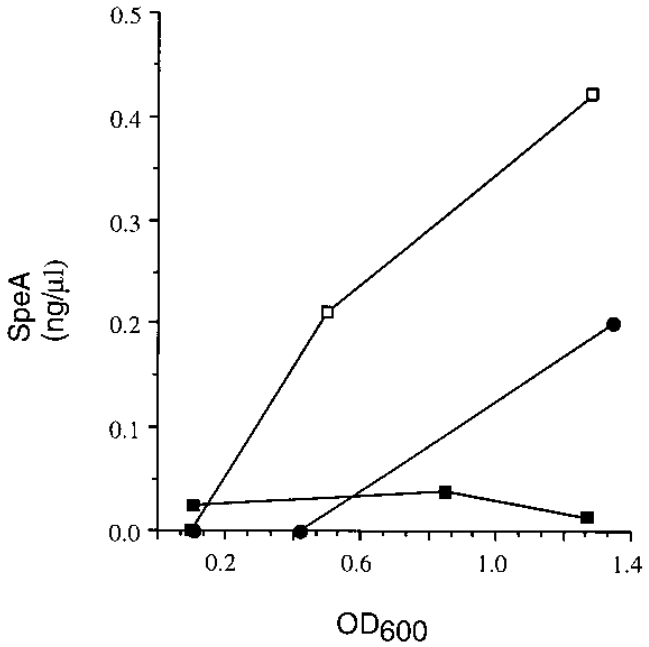

FIG. 4. Temperature regulation of *speA* in the T12-negative strain *S. pyogenes* ATCC 12204. *speA* was inserted into the streptococcal shuttle vector pLZ12-Km and electroporated into the *speA*-negative (T12-negative) strain ATCC 12204 to form ATCC 12204-*speA*. Cultures were grown at either 37 or 26°C, and aliquots taken at the indicated OD values were examined for SpeA by competitive ELISA. □, strain ATCC 12204-*speA* grown at 37°C; ●, strain 12204-*speA* grown at 26°C; ■, strain ATCC 12204 grown at 37°C.

to X-ray film, the Northern blot was stripped of the *speA* probe and hybridized with a DNA fragment homologous to a conserved region of *emm24* coding for the C and D repeats (Fig. 3A, bottom). Densitometric scans of the resultant autoradiographs indicate that there was a sixfold increase in *speA*-specific RNA at 37°C when compared to the level at 26°C. A 1.5-fold difference in the levels of the *emm* gene was detected (level at 37°C was higher than that at 26°C). The *emm* regulation is consistent with the observations of others (3), although it differs from the fourfold regulation of *emm* seen in some reports (10). No toxin-specific or M protein-specific mRNA was detected with RNA samples from *S. pyogenes* Ste.

Northern analysis was performed on RNA isolated from the SpeA-producing strain *S. pyogenes* NY5, and a densitometric scan of the resultant autoradiograph indicated that a 3.5-fold increase in expression was seen with growth at 37°C when compared to growth at 26°C (Fig. 3B). As an additional control, RNA from *S. pyogenes* Ros was hybridized to a *speB* gene probe, and equal amounts of *speB* hybridizing material were seen with RNA isolated from cells grown at both temperatures (Fig. 3C). The Northern analysis suggested that transcription of *speA* increased approximately four- to sixfold with the shift in temperature. This increase in transcription was not seen with either *emm3* or *speB*.

Effect of T12-encoded genes on *speA* regulation. If there is a specific regulator of *speA* expression, this protein could be host encoded or found on the toxin-encoding bacteriophage genome. In order to determine if there was temperature regulation of *speA* in the absence of other phage-encoded genes, *speA* was moved into the bacteriophage T12-negative, *speA*-negative *S. pyogenes* strain ATCC 12204 background. A DNA fragment containing *speA* and ca. 300 bp upstream of *speA* was amplified by PCR and inserted into the streptococcal shuttle vector pLZ12-Km at the *Bam*HI and *Pst*I sites, and the recombinant plasmid was moved by electroporation (4) into strain ATCC 12204.

Expression of SpeA from the wild-type *speA*-negative ATCC 12204 and the *speA*-positive transformant ATCC 12204 is shown in Fig. 4. In the transformant, there was a twofold higher expression of SpeA at 37°C when compared to expression at 26°C. There was no expression of SpeA seen with the wild-type ATCC 12204. Therefore, there was increased expression at the higher temperature, although not the three- to fourfold seen with *S. pyogenes* NY5 or Ros. This could be due to the greater than one per chromosome copy number of *speA* in the transformant. These data indicate that temperature regulation occurs in the absence of other phage-encoded genes.

In humans *S. pyogenes* is found either on the skin, in the throat, or associated with invasive infections. *S. pyogenes* infections with SpeA-producing strains can result in either scarlet fever (from pharyngeal infections) or the previously described STSS. For both types of infections the organism is at the body temperature of 37°C (or greater) and expression of the toxin occurs. Organisms living on the skin are at temperatures less than 37°C. These organisms do not have direct access to the bloodstream, and therefore there is no advantage for them to produce the toxin.

There are other temperature-regulated virulence genes, including invasion-related genes of the gram-negative *Shigella* spp. and the pyelonephritis-associated pili (pap) of *Escherichia coli* (9). Temperature-regulated genes can be controlled by gene products specific for that trait (18); by histone-like proteins, such as H-NS, that are involved in changes in DNA topology and associated with the temperature regulation of a number of unlinked, unrelated genes (7, 14); or by changes in local DNA structure (15). Some temperature-regulated genes are expressed (or repressed) in response to a number of other environmental factors, such as osmolarity and extracellular pH. We have been unable to identify any additional environmental factors that influence the production of SpeA. Future experiments will be to attempt to identify a possible regulator of *speA* expression.

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