Production of Stabilized Virulence Factor-Negative Variants by Group A Streptococci during Stationary Phase

B. A. B. Leonard, M. Woischnik and A. Podbielski

Production of Stabilized Virulence Factor-Negative Variants by Group A Streptococci during Stationary Phase

B. A. B. LEONARD,† M. WOISCHNIK, AND A. PODBIELSKI*
Department of Medical Microbiology and Hygiene, University of Ulm Clinic, 89081 Ulm, Germany

Received 8 January 1998/Returned for modification 9 April 1998/Accepted 4 May 1998

Many of the virulence factors associated with fulminant group A streptococci (GAS) infection are expressed under in vitro exponential growth conditions. However, the survival of GAS in tissue and intracellularly, as well as colonization of asymptomatic carriers, has been reported for GAS. The bacteria associated with these niches may encounter high-density, low-nutrient-flowthrough conditions that may more closely mimic in vitro stationary-phase conditions than exponential growth. Therefore, the behavior of GAS in stationary-phase culture was examined. We observed that after 24 h in stationary phase, GAS serotypes M49 and M2 developed an unstable colony dimorphism of typical large and atypical small colonies. Between days 4 and 5, we isolated stabilized atypical small colonies which remained stable for up to nine passages (approximately 200 generations) on fresh medium before fully reverting to the large-colony phenotype. Upon analysis, the small colonies showed no difference in cell number per colony, growth rate, survival in prolonged stationary-phase culture, or anti-biotic sensitivity. However, the small colonies showed decreased transcription of hyaluronic acid capsule, the global positive virulence factor regulator gene mga, the mga-regulated emm mRNA (M-protein structural gene), and speB (cysteine protease). Accordingly, the small colonies were completely sensitive in a traditional phagocytosis assay. The production of virulence factors and phagocytosis resistance of the small-colony isolates was recovered when, after several passages on fresh medium, the colony morphology began to revert.

Group A streptococci (GAS) are important gram-positive human pathogens, causing both mild disease (pharyngitis and impetigo), severe life-threatening infections (toxic shock-like syndrome), and post-infection sequelae (rheumatic fever and glomerulonephritis) (recently reviewed by references 12 and 56). Important virulence mechanisms in GAS include expression of genes which depend on the presence of the multiple gene activator, Mga (encoded by virR [55] and mry [40]) and Mga-independent genes. Mga-dependent factors include a number of cell surface binding proteins responsible for such traits as phagocytosis resistance (M and M-related proteins; for a review, see reference 28) and fibronectin binding (serum opacity factor [29, 48]), as well as inactivation of complement factors (C5a peptidase [7]). These Mga-dependent virulence factors are all monocistronically transcribed, and many of them are located together in the core vir regulon (9, 41). Transcription of genes in other chromosomal locations such as the cysteine protease gene speB can also be influenced by Mga, although it is not known whether this is a direct or indirect effect (45). SpeB has been found to cleave, digest, or completely degrade a number of bacterial and human proteins (3, 23, 26, 27, 58). The alteration in activity of these proteins caused by SpeB appears to be an important component of infection and outcome, since SpeB mutants are significantly reduced in virulence (34). There are also a number of virulence factors which are Mga independent, including capsule, streptokinase, streptolysin O (SLO), erythrogenic toxin SpeA, and DNase D (45). Production of the hyaluronic acid capsule plays a role in determining overall infectivity by its effect on such processes as phagocytosis resistance (13, 38, 49).

The colonization of asymptomatic carriers by GAS has also been reported, and the findings suggest that the associated bacteria do not elicit fulminant disease by rapid multiplication in the host and the production of host-damaging virulence factors (18). Instead, the bacteria may enter a more quiescent state. A number of observations suggest that intracellular GAS may represent such a reservoir. In the 1950s, L forms of GAS isolated from eukaryotic cell vacuoles were described (20, 25, 32, 50, 51). These L forms had reduced production of virulence factors such as streptokinase, hyaluronidase, and erythrogenic toxin and were suggested to be in a nonreplicative state (24, 22, 32). The L forms persisted in a mouse model for up to 52 weeks, where they eventually could induce a variety of systemic lesions in tissue (25). Recent studies confirm that GAS can invade eukaryotic cells (11, 19, 22, 30). An association of intracellular GAS with asymptomatic carriers and patients suffering from recurrent tonsillitis suggests that this intracellular state may be important in vivo (39). In vitro stationary-phase culture may more closely mimic the high-density, low-nutrient-flowthrough, low-replication-rate conditions of intracellular GAS than exponentially growing cells. Therefore, we examined the expression of representative Mga-dependent and -independent virulence factors by M-protein serotypes M49 and M2 GAS during prolonged stationary-phase cultivation. Approximately 24 h after the onset of stationary phase, a colony size dimorphism could be detected upon plating on fresh medium. After prolonged incubation in stationary phase (3 to 5 days), the GAS formed stabilized atypical small colonies which were found to have reduced or no expression of Mga, M protein, SpeB, and capsule but unaffected and slightly increased levels of the housekeeping gene recA and the SLO hemolysin, respectively. Consistent with loss of expression of many important virulence factors, the stabilized small colony cells were completely sensitive to phagocy-
tosis. The atypical small colonies were stable for approximately 110 to 200 generations in fresh media before reverting to the more typical large colonies accompanied by a recovery of virulence factor production and resistance to phagocytosis.

MATERIALS AND METHODS

Strains and medium. GAS strains used in this study were serotype M49 strain CS101 (kindly provided by P. Cleary, Minneapolis, Minn.) and serotype M2 strain T2/44/RB4/119 (obtained from D. Johnson, WHO Reference Library, Minneapolis, Minn.).

Designation and scoring of atypical small colonies. GAS strains were grown in 5 ml of Todd-Hewitt yeast extract broth (THY) or on THY agar at 37°C in 5% CO2. At approximately the same time everyday (±3 h), aliquots were withdrawn, diluted in fresh THY broth, and plated on THY agar. Atypical small colonies were those that appeared to be reduced in size by at least 50% and were no longer glossy. Frequency was calculated by dividing the number of small colonies by the total number of colonies. The frequency is the average of at least three independent experiments. Stability of the small colonies was assessed by serial transfer of the small colonies on Mueller-Hinton sheep blood agar (MH-SBA) plates. Stabilized small colonies were those colonies which retained the small-colony phenotype through at least two passages on MH-SBA.

Growth measurements. The number of cells per stabilized small and typical colonies was determined by plating the cells on MH-SBA and extracting single colonies from the agar plate that went the entire depth of the agar. The loop containing the colony was placed in a microcentrifuge tube, 1 ml of THY broth was added, and the colony was vortexed at full speed for 1 min. Cells per ml were determined by serial dilution and plating on THY agar.

Growth curves were generated by diluting an overnight culture (12 to 15 h, 37°C, 5% CO2 in THY broth) 1:10 in fresh THY broth and measuring cell optical density at 600 nm (OD600) (Ultratech 3000; Pharmacia Biotech, Freiburg, Germany). Viable cell numbers per OD unit were determined by dilution plating of cultures at OD600 of 0.5 and 1.0. This method was used to assess whether the OD600 for small and typical colonies represented approximately the same number of cells.

The ability of small colonies to survive in stationary phase was determined by inoculation of 5-ml THY cultures with passage 3 stabilized small colonies followed by prolonged incubation at 37°C and 5% CO2. Aliquots were removed at approximately the same time (±3 h) every day, diluted, and plated on THY agar. The stability of the small colonies was assessed by sequential passages on MH-SBA (once every 24 h). Using an average chain length of four (so that each colony originates from an average of four cells) and the fact that extracted colonies had 3 $\times$ 10$^4$ to 4 $\times$ 10$^4$ CFU, it was calculated that one passage on MH-SBA represents approximately 22 generations.

Antibiotic resistance. Small and typical cells were plated to confluency on MH-SBA and tested for antibiotic sensitivity with E-test strips as instructed by the manufacturer (Difco Laboratories, Augsburg, Germany).

Quantification of hyaluronic acid. Hyaluronic acid was isolated from the culture supernatants of various strains after overnight incubation of the GAS at 37°C in 5% CO2 in 10 ml of chemically defined medium (CDM) (57). Cells were removed by centrifugation at 2,500 × g for 10 min, and the supernatant was filtered. The supernatant was adjusted to a final concentration of 0.5% sodium decylpyridinium chloride (Fluka, Neu-Ulm, Germany) and incubated for 1 h at 37°C. The precipitant was centrifuged at 4,000 × g for 30 min and resuspended in 2 ml of 0.5 M NaCl. Three volumes of ethanol was added, and the hyaluronic acid was precipitated by incubating at −20°C overnight. The hyaluronic acid was redissolved in 2 ml of 0.5 M NaCl. Three volumes of ethanol was added, and the hyaluronic acid was precipitated by incubating at −20°C overnight. The hyaluronic acid was redissolved in 2 ml of 0.5 M NaCl. Three volumes of ethanol was added, and the hyaluronic acid was precipitated by incubating at −20°C overnight. The hyaluronic acid was redissolved in 2 ml of 0.5 M NaCl. Three volumes of ethanol was added, and the hyaluronic acid was precipitated by incubating at −20°C overnight. The hyaluronic acid was redissolved in 2 ml of 0.5 M NaCl. Three volumes of ethanol was added, and the hyaluronic acid was precipitated by incubating at −20°C overnight. The hyaluronic acid was redissolved in 2 ml of 0.5 M NaCl. Three volumes of ethanol was added, and the hyaluronic acid was precipitated by incubating at −20°C overnight. The hyaluronic acid was redissolved in 2 ml of 0.5 M NaCl. Three volumes of ethanol was added, and the hyaluronic acid was precipitated by incubating at −20°C overnight. The hyaluronic acid was redissolved(51,269),(946,978)

Detection of SpeB by SDS-PAGE. SpeB production was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) analysis of culture supernatants. The different strains were grown overnight in 30 ml of THY broth at 37°C in 5% CO2 (approximately 16 h). Cells were harvested by centrifugation at 2,500 rpm for 10 min. The supernatant was filtered, sterilized, adjusted to 15% trichloroacetic acid (Fluka), and let stand at 4°C for 1 h. The precipitated proteins were harvested by centrifugation at 5,000 × g for 30 min, washed twice with cold acetone (~−20°C), and finally boiled for 5 min in 30 μl of Laemmli SDS-PAGE buffer (2). Proteins were then electrophoresed through an SDS-12.5% minigel (Biometra, Goettingen, Germany) and stained with Coomassie blue. Prestained Bio-Rad (Hercules, Calif.) SDS-PAGE standards (low range) were used as molecular weight markers.

mRNA detection and comparison. RNA was prepared by growing cells overnight in THY broth at 37°C in 5% CO2, for 12 to 15 h. On the following day, the cells were diluted 1:2 in fresh THY broth and incubation was continued for another 4 h under the same conditions. These cells represent late-log-phase cultures with a final OD600 of approximately 1.0. RNA was isolated by the hot phenol extraction method of Shaw and Clewell (53). The isolated RNA was quantified by determining the OD260 of the sample. Denaturing agarose gel electrophoresis and semiquantitative Northern blotting were done on dilutions of the RNA representing 40 to 1.25 μg of total RNA, following the method of Podbielski et al. (42). Consistency in gel loading was confirmed by visual inspection of ethidium bromide-stained gels. Digoxigenin (DIG)-labeled PCR product probes were generated as previously described (42), using 1 μg of GAS M49 chromosomal template prepared by the method of Anderson and McKay (1) and 100 pmol of each primer. Hybridization and disodium-3-(4-methoxyphenyl)-1,2-dioxetane-3'-2'-(5-chloro)tricyclo[3.3.1.3$^3$]decan-4-ylphenyl phosphate (CSPD) visualization were accomplished as previously described (42).

The RNA for each assay was freshly prepared. The blots represent the results of at least two independent experiments using two different stabilized small or typical colonies isolated during independent experiments.

Determination of phagocytosis resistance. Phagocytosis resistance was determined by the method of de Malmanche and Martin (14).

RESULTS

GAS forms small colonies after prolonged growth in stationary phase. During studies on stationary-phase GAS, it was observed that the cells demonstrate colony dimorphism between typical large colonies and atypical small colonies after prolonged incubation. Wild-type M2 and M49 GAS were grown in complex medium (THY broth). After reaching stationary phase, cells were incubated without additional nutrients under the same conditions, and samples were periodically removed and plated on THY agar. Approximately 24 h after the onset of stationary phase, we observed the formation of atypical small colonies on the THY plates at a frequency of 1 $\times$ 10$^{-1}$ to 5 $\times$ 10$^{-1}$ (Fig. 1). These colonies were significantly (at least 50%) smaller than the typical large colonies and appeared drier. To determine whether the small colonies represented slow-growing cells that would eventually form colonies of the same size, the plates were incubated for a further 24 h (48 h in total). The small colonies did not continue to grow after the large colonies had ceased to grow, suggesting that the small colonies were not a result of delayed or slower growth of the initial cells. The small colonies isolated 24 h after exponential phase reverted to large colonies upon subsequent passage on THY agar or MH-SBA. Three to five days after exponential phase, 20 to

FIG. 1. Formation of stable atypical small colonies by GAS serotype M2 and M49. GAS serotypes M2 and M49 were incubated at 37°C in 5% CO2 for a number of days in THY broth without addition of fresh medium. At various days after inoculation, aliquots were withdrawn, plated on THY agar, and incubated at 37°C in 5% CO2. The column indicated by “strain on THY” is representative of the colony dimorphism that was obtained 1 day after exponential growth and continued throughout stationary-phase incubation. To check for formation of stable atypical small isolates, representative colonies from the resulting THY plate were transferred to MH-SBA and incubated under identical conditions. The passage number is the number of times the small-colony isolates from the THY plates were sequentially passed on MH-SBA. The results are representative of those obtained with at least three independently derived stabilized small colonies.
from a small isolate or typical isolate. No significant difference in corresponding OD values and viable counts were obtained between cell cultures derived from small and typical isolates. The cell phenotype was maintained during the incubation (data not shown).

Additionally, SCVs have been shown to be refractile to antibiotic treatment (8). The small and large colonies showed no significant differences in antibiotic sensitivity as determined by the E-test (Table 1).

**Production of virulence factors in small colonies.** Since the M49 strains produce a large amount of hyaluronic acid (unpublished observations), one possible explanation for the smaller and less glossy appearance of the atypical colonies was the loss the capsule production. By quantitative capsule assay, it was found that passage 3 stabilized small colonies produced significantly less capsule after overnight growth in CDM than the corresponding wild-type cells (see Fig. 4). In accordance with the lack of detectable capsule, no has capsule synthesis operon expression in late-logarithmic-phase cultures inoculated with passage 3 stabilized small colonies could be detected by Northern blotting with an internal DIG-DUTP-labeled has internal probe (Fig. 3). This reduction in capsule production may account in part or entirely for the size of the small colonies, since has mutants exhibit a similar colony morphology (Fig. 4).

The expression of the global positive virulence regulator, Mga, as well as Mga-dependent virulence factors was also determined in late-exponential-phase cultures inoculated with passage 3 typical and small colonies. DIG-labeled internal PCR probes to the M protein (emm), Mga (mga), and SpeB (spdB) genes were generated (45). The approximate level of mRNA expression was determined by Northern blotting of twofold serial dilutions of total RNA extracted from typical and stabilized small-colony isolates. The sizes of the transcripts were in agreement with previously reported molecular masses (45). mga expression, and, accordingly, expression of the Mga-dependent speB and emm genes, was found to be significantly reduced (Fig. 3). While transcription of mga and the Mga-dependent emm gene were reduced equivalently, transcription of the Mga-dependent speB gene and production of SpeB protein were abolished, as determined by Northern blotting (Fig. 3) and SDS-PAGE (data not shown).

To determine whether the general mRNA transcription rates were altered in the stabilized small colonies, recA expression was measured in late-exponential-phase cultures inoculated with passage 3 typical and stabilized small colonies. Expression of this gene was previously used as a housekeeping gene marker by McIver et al. (36). The recA gene was found to

### Table 1. Antibiotic sensitivity of typical and atypical small colonies

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Typical</th>
<th>Small</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefotaxime</td>
<td>0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>0.19</td>
<td>0.094</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.19</td>
<td>0.25</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>0.032</td>
<td>0.064</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>48</td>
<td>32</td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>4.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Penicillin</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>1.0</td>
<td>0.75</td>
</tr>
</tbody>
</table>

* Determined by the E-test according to the manufacturer’s instructions.
be expressed at equal levels per microgram of total RNA in the stabilized small colonies and typical colonies (Fig. 3). In addition, expression of the Mga-independent hemolysin gene, slo, appeared to be slightly increased in the stabilized small colonies (Fig. 3). All mRNA expression patterns tested were the same for at least two independently derived stabilized small colonies.

**Phagocytosis sensitivity of small colonies.** GAS resistance to phagocytosis in the classical phagocytosis assay is due to combined abilities of a strain to obtain the essential nutrients leading to multiplication in blood and to prevent binding to phagocytes (44, 52). Since production of both M protein and capsule has been shown to be important for phagocytosis resistance (13, 38, 44, 49), the phagocytosis resistance of the atypical small M49-derived colonies was determined (Table 2). The number of CFU which could be isolated after incubation of passage 3 stabilized small colonies in blood was greatly reduced (Table 2), suggesting the cells did not multiply in blood and were almost completely sensitive to killing by phagocytes.

**Recovery of virulence factor production upon reversion to large colonies.** After approximately 110 to 200 generations (five to nine sequential passages) on MH-SBA, the small colonies began to revert to the large-colony phenotype, showing stronger hemolysis and larger, glossier colonies (Fig. 1). Consistent with the colony morphology, production of the emm, has, and mga messages recovered (Fig. 5). The cells also became resistant to phagocytosis (Table 2), and in passage 5, cultures which generally had a mix of small and large colonies, an intermediate level of phagocytosis resistance was found, with only large colonies being recovered from the phagocytosis resistance assay (Table 2).

**DISCUSSION**

The only known reservoir for GAS is the human host. Many of the virulence factors exhibited by GAS have been shown to be expressed during active growth, and their expression is down-regulated upon entry of the cells into stationary phase. This has important implications for in vivo expression of virulence factors during infection. During fulminant infection, the bacteria may be rapidly multiplying under relatively low densities with sufficient nutrients, much like in vitro exponential growth conditions. Many of the virulence factor genes are under the control of the global positive virulence factor regulator, Mga (9, 40, 45). Mga-controlled genes have been found to be regulated by pH, temperature, pCO₂, Fe²⁺ concentrations, exponential growth phase, and late logarithmic growth phase (6, 36, 37, 43). The combination of the types of virulence factors regulated and the inducing conditions have led investigators to propose that Mga-regulated genes promote bacterial invasion into deep tissue (6).

After tissue penetration and/or subsequent invasion into the local eukaryotic cells, GAS may no longer be under ideal growth conditions. Instead, at infection foci in tissue or intracellularly, the cells may encounter high-density, low-nutrient-flowthrough conditions that may more closely mimic stationary-phase conditions than exponential growth. The response of the bacteria to stationary-phase conditions may be equally

**TABLE 2. Phagocytosis sensitivity**a of typical and atypical small M49-derived colonies

<table>
<thead>
<tr>
<th>M49 colony type</th>
<th>Passage no.</th>
<th>Multiplication factor (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Typical</td>
<td>3</td>
<td>23.6 ± 5.1</td>
</tr>
<tr>
<td>Small</td>
<td>3</td>
<td>0.04 ± 0.035</td>
</tr>
<tr>
<td>Typical</td>
<td>5</td>
<td>20.3 ± 1.45</td>
</tr>
<tr>
<td>Small</td>
<td>5a</td>
<td>8.1 ± 2.5</td>
</tr>
</tbody>
</table>

a Determined by the direct bactericidal assay.

b Approximately 50% of the colonies obtained upon plating of the passage 5 small isolate had reverted to typical large colonies. Greater than >95% of the colonies recovered after the phagocytosis resistance assay formed typical-size colonies on THY plates.
important in their overall virulence. It is possible that these types of conditions can induce alternate states of GAS. For example, the bacteria isolated from asymptomatic carriers have been shown to express less M protein, and intracellular L forms of GAS which express reduced levels of streptokinase, hyaluronidase, and erythrogenic toxin have been described (24). The down-regulation of virulence factors as a part of intracellular survival could be logical to prevent both eukaryotic cell activation and death (15). To examine the response of GAS to niches with high-density, low-nutrient conditions, we examined the expression of virulence factors by GAS during prolonged stationary-phase incubation.

In these studies, we found that GAS form small colonies after 24 h in stationary-phase culture. The frequency of small-colony formation ranged from $5 \times 10^{-1}$ to $1 \times 10^{-1}$, with approximately 20 to 50% of the small colonies remaining stable 3 to 5 days after onset of stationary phase (stabilized small colonies). This phenomenon could be observed for both M49 and M2 serotypes of GAS, suggesting that the phenomenon was not M-type specific.

One striking feature of the small colonies was their dry appearance. Since capsule production results in the formation of glossy colonies, the capsule production of the small colonies was quantitated on both the hyaluronic acid and mRNA levels. The stabilized small colonies produced no detectable hyaluronic acid and no detectable has capsule synthesis operon mRNA. This finding indicates that the loss of capsule results from shutdown of has transcription. A number of observations support the conclusion that the small appearance of the atypical colonies probably resulted from loss of capsule expression and not a significant alteration in metabolic activity. First, the size of GAS colonies appeared to correlate with capsule production; those M-protein serotypes producing more capsule are generally larger (unpublished observations), and capsule synthetase (has) mutants have the same general appearance as the atypical small colonies. The stabilized small colonies had the same number of cells per colony, as judged by extraction of the colonies and subsequent viable plate counting. This result could be affected somewhat by chain length, but upon Gram staining, the chain lengths of the bacteria isolated from typical and small colonies showed no significant difference (data not shown). Prolonged incubation of the plates did not result in the further growth of the small colonies, and the two colony types had indistinguishable growth kinetics in fresh liquid medium with the same viable plate counts per unit of OD. This finding suggests that the colonies were not simply growing slower due to a general repression of a nutrient acquisition system that takes many generations to recover.

Furthermore, transcription of the housekeeping gene recA (36) or the virulence factor gene slo was unaffected or slightly increased, respectively, in late-logarithmic-phase cultures derived from stabilized small colonies. This finding indicates a specific effect on the expression of certain virulence factors and not a general repression of transcription. The continued expression of slo should be noted since the stabilized small colonies exhibit on overall weaker hemolysis on MH-SBA. GAS have been reported to produce both SLS and SLO hemolysins (28). The expression of a gene linked to SLS production gene (4) was reduced more than eightfold in the stabilized small colonies (data not shown) and probably accounts for the observed reduction in hemolysis.

The transcription of the mga global regulatory gene and Mga-dependent virulence factors was also found to be reduced in the atypical small colonies. Due to the time required for RNA preparation for Northern blotting, it was not possible to distinguish whether the observed decrease is due to alterations in transcription rate or message stability. It is interesting that while the rates of transcription or stability of mga and the Mga-dependent emm transcript were reduced in the small colonies, the Mga-dependent speB mRNA was decreased to undetectable levels and no SpeB protein could be detected by SDS-PAGE. This implies a proportionally greater reduction in speB mRNA than in mga or the mga-regulated emm transcript. While there are a number of possible explanations for this observation, the most probable is the changes in expression or activity of a second regulatory pathway which is responsible for apparently complete shutdown of speB.

The direct bactericidal assay measures a number of parameters, including the ability of an organism to multiply extracellularly in blood, bind to phagocytes, and resist killing by granulocytes (14, 52). Therefore, this multiparameter assay could be considered to be a measurement of overall survival capacity during septicemia. Both the production of capsule and the production of M-protein have been shown to play a role in phagocytosis resistance (13, 38, 45, 49; for recent review on the role of M protein, see reference 28). Consistent with the lack of important virulence factor expression in the atypical small-colony-forming isolates, these bacteria were almost completely sensitive to phagocytosis.
The small-colony phenotype could be reversed by subsequent passage for a minimum of 110 generations on MH-SBA or THY medium. Concomitant with the reversion to typical passage for a minimum of 110 generations on MH-SBA, the phenotype is not stable smaller compact colonies are the same as the unstable smaller compact colonies were sensitive to penicillin, suggesting that they still contained an intact cell wall and, additionally, do not represent a highly antibiotic resistant form of the bacteria such as the SCVs of S. aureus.

ACKNOWLEDGMENTS

The work of A.P. was supported by DFG grant Po391/6-2. B.A.B.L. was an Alexander von Humboldt Foundation Fellow (IV-USA/1089266).

REFERENCES

and pathological effect of streptococcal L-forms in vivo. INSERM 65:247–258.
19-
20-
49. Rakonjac, J. V., J. C. Robbins, and V. A. Fischetti. 1995. DNA sequence of the serum opacity factor of group A streptococci: identification of a fibronec-
21-
51. Schmitt-Slomska, J., A. Boue, and R. Caravano. 1972. Induction of L-vari-
22-
23-