The Ability of *Aneurinibacillus migulanus* (Bacillus brevis) To Produce the Antibiotic Gramicidin S Is Correlated with Phenotype Variation

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Phenotype instability of bacterial strains can cause significant problems in biotechnological applications, since industrially useful properties may be lost. Here we report such degenerative dissociation for *Aneurinibacillus migulanus* (formerly known as *Bacillus brevis*) an established producer of the antimicrobial peptide gramicidin S (GS). Phenotypic variations within and between various strains maintained in different culture collections are demonstrated. The type strain, ATCC 9999, consists of six colony morphology variants, R, RC, RP, RT, SC, and SP, which were isolated and characterized as pure cultures. Correlations between colony morphology, growth, GS production, spore formation, and resistance to their own antimicrobial peptide were established in this study. We found the original R form to be the best producer, followed by RC, RP, and RT, while SC and SP yielded no GS at all. Currently available ATCC 9999T contains only 2% of the original R producer and is dominated by the newly described phenotypes RC and RP. No original R form is detected in the nominally equivalent strain DSM 2895T (=ATCC 9999T), which grows only as SC and SP phenotypes and has thus completely lost its value as a peptide producer. Two other strains from the same collection, DSM 5668 and DSM 5759, contain the unproductive SC variant and the GS-producing RC form, respectively. We describe the growth and maintenance conditions that stabilize certain colony phenotypes and reduce the degree of degenerative dissociation, thus providing a recommendation for how to revert the nonproducing smooth phenotypes to the valuable GS-producing rough ones.

Gramicidin S (GS) is a cyclic decapeptide ([FPVOLFPVOL]_{cyc}) which has been used prominently as an antibiotic in local applications since 1944 (13, 14). The peptide exerts a wide range of antimicrobial effects, including activity against gram-positive and gram-negative bacteria, viruses, fungi, and single-cell pathogenic eukaryotes (5, 13, 19, 22, 23, 52). The primary molecular target of GS is the cellular membrane, though the detailed mechanism of its cytotoxic activity is still a matter of debate (20, 29, 32, 49). Due to its high stability and simple structure, GS is also commonly used as a reference compound in mass spectrometry and nuclear magnetic resonance spectroscopy, and it has served as a model peptide for studying functional mechanisms of membrane-active peptides (9, 16, 18, 30, 37, 41, 42, 50). Unfortunately, however, chemically pure GS is no longer available from any open commercial sources (for example, Sigma-Aldrich stopped placing it in their catalogue as of 2000).

The original bacterial strain that could produce GS was isolated in 1942 from Russian garden soil among several hundreds of isolates. It was the only effective antagonist against pathogenic *Staphylococcus* strains and was described as *Bacillus brevis* var. G.B. (14). This commercially valuable GS producer was then maintained over several decades in various culture collections. Initially, *B. brevis* var. G.B. was delivered to the National Centre of Type Cultures (London, United Kingdom), from which it was obtained by the American Type Culture Collection (ATCC, Manassas, VA), where it was deposited as *B. brevis* ATCC 9999. The Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) received and preserved this strain as DSM 2895. DSMZ also maintains two other strains shown to produce GS, namely, *B. brevis* DSM 5759 and *B. brevis* DSM 5668.

Over recent decades, the taxonomic status of the GS producer has changed several times; therefore, it appears in the literature under different names (most often still *Bacillus brevis*). Initially, the bacterium was referred as *B. brevis* Migula 1900. Results of further polyphasic taxonomic studies of 35 different *B. brevis* strains then led to the reclassification of ATCC 9999 as a type strain (=ATCC 9999T) of a new species: *Bacillus migulanus* sp. nov. (38). In 1996, using gene sequence alignment of 16S rRNA, ATCC 9999T was reclassified: *Aneurinibacillus migulanus* comb. nov. (34). Based on DNA-DNA hybridization with DSM 2895T, the two other GS-producing strains from DSMZ (DSM 5668 and DSM 5759) were assigned to *A. migulanus* as well (15).

In early applications for industrial GS production, a colony morphology variability of the original isolate was discovered (54, 55). *B. brevis* var. G.B. was initially described as having a rough (R) colony morphology phenotype (rough, dull, dense, rugose colonies 3 to 5 mm in diameter, with undulated edges), which can spontaneously dissociate into a smooth (S) form (smooth, glistening, convex, soft, whitish, 3 to 5 mm). By comparing the morphological, cultural, physiological, and biochemical properties of spontaneous dissociants, in total four distinct variants were identified: R, S, P(−) (planar, dull, dense, soft, with undulated edges, 10 to 12 mm), and P(+) (planar, translucent, dull, soft, with undulated edges). Only the R and P(+) forms were shown to produce GS (54). Several environ-
mental factors were found to promote dissociation or to stab-
libilize certain phenotypes of B. brevis var. G.B. For example, exposure to UV irradiation or the addition of β-alanine, phenylalanine, proline, tyrosine, methionine, and threonine in li-
muid medium was shown to stabilize the R form, while the addition of glutamic and aspartic acid stabilized the P phenotype, and the presence of histidine and arginine facilitated an R-to-S dissociation (36, 53).

Bacterial dissociation is a well-known phenomenon (6, 43) and may often lead to an unfortunate loss of ability to produce desired compounds. For instance, recent cultures of B. brevis DSM 362 (=ATCC 8185), known to produce gramicidin A and tyrocidine A, have lost their ability to make these antimicrobial peptides (45). Similarly, degenerative dissociation was ob-
erved in Clostridium acetobutylicum, which is industrially used to produce acetic acid and butanol. For ATCC 4259, ATCC 824, and some other soil isolates, it was demonstrated that sponta-
eneous changes in colony morphology are associated with this loss of solvent production (1). Interestingly, degeneration of these cultures was usually accompanied by loss of the ability to sporulate. By investigating such bacterial dissociation and identifying reliable culture properties (e.g., colony morphology and/or character of sporulation), which correlate with produc-
tivity, it should be possible to prevent the loss of useful features during strain maintenance.

Here, we analyze the degenerative diversity of bacterial strains that have been established as GS producers, in order to select the best one for large-scale production of this anti-
microbial peptide. We describe a wide repertoire of observed colony morphology variants and characterize them in detail. The productivity of each form is systematically quantified and correlated with phenotype. In addition, the respective growth characteristics, the morphology of the vegetative cells and spores, the sporulation activity, and the tolerance for externally added GS are discussed.

MATERIALS AND METHODS

Materials. Bacto tryptone with 4 to 6% amino nitrogen (AN) and Difco Noble agar were purchased from BD Diagnostic System (Sparks, MD); beef extract powder and GS (note that the peptide is no longer available; formerly catalog no. G-0000) were obtained from Sigma (St. Louis, MO). Yeast extract with 5% AN and agar for microbiology were from Fluka BioChemika (Buchs, Switzerland). Deminerlized water purified with a Milli-Q Biocell system from Millipore (Bed-
ford, MA) was used. Inorganic salts, solvents, and other chemicals were of the highest quality available.

Bacterial strains. A. migulanus ATCC 9999 was obtained from the ATCC. The same strain deposited as A. migulanus DSM 2855, along with two other nominal GS producers, A. migulanus DSM 5668 and DSM 5759, were received from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany).

Cultivation media. Three complex media with different AN contents were used. All media were adjusted to a pH of 7.4 at 25°C (with 1 M NaOH) before autoclaving. First, for the preparation of spores, NBYS medium was used. For this medium, the AN content is estimated to be around 50 mg/dl. The compos-
ition is as follows: Bacto tryptone, 5 g/liter; beef extract powder, 3 g/liter; yeast extract, 5 g/liter; MgCl2 • 6H2O, 0.2 g/liter; CaCl2 • 2H2O, 0.1 g/liter; MnCl2 • 4H2O, 10 mg/ml; and FeCl3 • 4H2O, 0.2 mg/ml. Second, to examine the homogeneity of the colony morphology phenotypes, fresh agar plates with solid LB medium were employed. In this case AN is determined to be 100 mg/dl. LBY medium is as follows: Bacto tryptone, 10 g/liter; yeast extract, 10 g/liter; NaCl, 0.5 g/liter; microbial agar, 25 to 30 g/liter. Note that an elevated concentration of the yeast extract and microbial agar used here is necessary to obtain distinguishable colonies (46, 54, 55). When cultivated on standard nutrient agar, A. migulanus demonstrates an irregular, entire, smooth, and glis-
tening growth. This growth behavior was found to obscure the correct evaluation of colony morphology. Finally, to estimate the overproduction of GS by each colony morphology phenotype, AN-rich (500 mg/dl) YP medium was used. This medium is described as the most effective complex medium for GS biosynthesis (25). YP is as follows: Bacto tryptone, 50 g/liter, and yeast extract, 50 g/liter. For this medium, autoclaving was performed at 121°C for 50 min.

Production of GS. For sporulation, 40 ml NBYS medium in 350-ml Erlen-
meier flasks was inoculated with material obtained from the culture collections or, for isolation of pure cultures, with a single morphologically distinct colony. Cultures were incubated for 2 days at 40°C and 220 rpm in a Minintron shaker incubator (Infors AG, Bottmingen, Germany). Cellular morphology and sporulation formation were examined after 16 h of growth under an Axioskop 40 light microscope (Carl Zeiss Lichtmikroskopie, Göttingen, Germany) equipped with a phase-contrast objective (A-Plan 100×) and a PowerShot G5 digital camera (Canon, Tokyo, Japan). The sizes of spores and vegetative cells were measured with an appropriate micrometer. To monitor the growth of cul-
tures, the optical density at 660 nm (OD660) was measured every 2 to 4 h on a SmartSpecPlus UV-visible spectrum spectrophotometer from Bio-Rad Laboratories ( Hercules, CA).

Spores were harvested by centrifugation at 9,000 × g at 4°C for 20 min (Sigma 3-18K centrifuge; Sigma Labofzentrifugen GmbH, Osterode, Germany). The obtained pellets were washed (twice with 40 ml each time) and resuspended (10 ml) in sterile ice-cold Milli-Q water. To inactivate the vegetative cells, spore suspensions were incubated at 80°C for 15 min, followed by chilling on ice and dilution to 40 ml with ice-cold Milli-Q water. Most of the cell debris was sepa-
rated from the spores by centrifugation (12 min, 250 × g, 4°C). The soft white pellet of spores was resuspended in 10 ml ice-cold Milli-Q water and stored at 4°C. Additionally, spore suspensions were preserved as 30% glycerol stocks at −20°C. The sporulation activity was quantified by counting colonies, after appropri-
ately diluted spore suspensions were plated out on LBY agar. To activate spore outgrowth, 200-μl aliquots of the initial dilutions were incubated at 80°C for 15 min. The activity was calculated as CFU/ml of the initial volume of the spore-
forming culture, or as CFU/g of cells (dry weight [DCW]; 1 g DCW is equivalent to an OD660 of 2.5, according to reference 25).

Colonies morphology. The intrastain diversity, colony morphology and homo-
Geneity of the populations isolated in pure cultures were evaluated after com-
plete expression of the phenotypes on LBY agar, and they were documented under a low-power Hund Wetzlar SM33 microscope (Wetzlar, Germany) equipped with a PowerShot G5 digital camera (Canon, Tokyo, Japan).

Production of GS and extraction. After the cultures had reached OD660 values of 2 to 3.1 ml of culture was removed from the flasks every 2 to 4 h to determine the yield of GS. Due to the intracellular localization of GS (25, 48), the cells were immediately centrifuged (7,500 × g, 15 min, 4°C). The pellet was resuspended in 1 ml of the pre-extraction solution (150 mM NaCl, 20 mM HCl) and incubated at 80°C for 15 min to facilitate the extraction of GS. Subsequently, the suspen-
sions were diluted 1:1 with absolute ethanol, and GS was extracted by agitating the suspension for 1 h at room temperature. The cell debris was separated by centrifugation, and the amount of GS in the ethanolic extract was determined in two ways. For the microbiological assay of GS activity, one-fifth of the total extract was taken, and the remainder was used for GS quantification by high-
performance liquid chromatography (HPLC).

Growth inhibition by GS. Resistance of the A. migulanus bacteria themselves to the antibiotic peptide GS was evaluated by determining the MICs in a stan-
dard dilution assay (3), using microtiter plates from Nunc GmbH & Co KG (Wiesbaden, Germany). Precultures were obtained in standard LB medium (pH 7.4 at 25°C) by inoculation with spore suspensions (final concentration, 103 to 104 CFU/ml). Two GS stock solutions in 50% ethanol were prepared, with 3.2 g/liter and with 0.32 g/liter. Serial twofold dilutions of these stocks were used to test the ranges from 800 to 200 mg/liter and from 80 to 1.25 mg/liter. All experiments were performed four times for statistical accuracy, including the positive (no-
peptide) and negative (uninoculated) controls. The plates were incubated for 24 h at 37°C, and MICs were determined as the lowest concentration of GS that reduced growth by more than 50%.

Quantification of GS by bioassay. The yield of GS was determined one way, using a four plate diffusion bioassay with Bacillus subtilis ATCC 6633 as a test organ.

Lab cultures were constructed with commercial GS. The range of GS concentrations in the 50%-ethanolic extract showed a linear concentration dependence of the activity between 25 and 250 mg/liter. The initial GS extract was diluted twice, five time, and ten times, and 50 μl of each dilution were
be conveniently compared and contrasted (Fig. 1). Altogether, colonies grow side by side, these morphological variations can be absolute equivalents, only one was able to produce the peptide. In order to understand the cause of this discrepancy, we performed detailed morphological studies of the different strains.

**Morphological diversity of A. miguulanus colonies.** First-generation spores from the four strains were plated on LBY agar to analyze their colony morphology. The strains exhibited different colony morphology phenotypes, which coexisted in the same culture. On plates with a high growth density, where the colonies grow side by side, these morphological variations can be conveniently compared and contrasted (Fig. 1). Altogether, dissociation into six distinct colony morphology variants was observed.

In the ATCC 9999T culture, the majority of colonies were 5 to 7 mm in diameter, dull, and soft and exhibited a pronounced convex elevation in the center (type I) (Fig. 1A). The second most abundant colony type was 8 to 10 mm in diameter, dull, flat, soft, and rough, with a small point-like elevation in the center (type II) (Fig. 1B). Types I and II together accounted for 86% of the strain population in ATCC 9999T. Some 7% of the colonies were rough but were also glistening, flat, and translucent, and these were designated type III (Fig. 1B). Nearly 5% of the colonies also retained umbonate elevations but were smaller (3 to 5 mm), smooth, and glistening (type IV) (Fig. 1C). About 2% of the colonies were also small (3 to 5 mm), rough, raised, dull, and dense (type V) (Fig. 1A and 1C). Only 2 colonies out of 1,000 were smooth, glistening, and slightly raised and had an average diameter of 8 to 12 mm (type VI) (Fig. 1D).

All of these colony forms were found to coexist in ATCC 9999T (Table 1). The other type strain, DSM 2895T, contained a mixture only of types IV and VI; in DSM 5668 we found exclusively colonies of type IV. The GS-producing strain DSM 5759 mostly contained colonies similar to type I, but unlike those from ATCC 9999T, they were entirely rough.

### Results

**Production of GS by different strains of A. miguulanus.** Four strains of *A. miguulanus* which have been extensively documented as producers of GS were evaluated here with regard to their quantitative yield of peptide biosynthesis. The results are summarized in Table 1, which shows that only ATCC 9999T and DSM 5759 are able to synthesize the peptide, whereas DSM 2895T and DSM 5668 gave zero yield. Using an AN-rich YP medium, we obtained higher absolute yields of GS than in a sporulation-promoting NBYS medium, as expected. When going from NBYS to YP, the specific and absolute activities of the DSM 5759 culture increased more than those of ATCC 9999T, which we distinguish as types I through VI.

**Quantification of GS by HPLC.** The remaining portions of the initial cell extracts (see above) were lyophilized and reextracted in 1 to 2 ml absolute ethanol by incubation at 60°C for 10 min. The solvent was evaporated in a Univapo 100H (Froebel, Lindau, Germany), and the dry material was dissolved in 80 µl of 50% ethanol to obtain a concentration that was 10-fold higher than in the cell culture. Analytical HPLC was performed on a chromatographic system from Jasco (Tokyo, Japan) equipped with a diode array detector. A reverse-phase C18 column (4.6 by 250 mm) from Grace (Deerfield, IL) was employed, with a linear water-acetonitrile gradient (relevant part of the gradient, 45 to 90% B in 15 min) at 35°C. Solvent A was 10% acetonitrile in 5 mM HCl; solvent B was 90% acetonitrile in 5 mM HCl. For all samples, a constant injection volume of 20 µl was applied. The peptide was detected at 257 nm, which is the characteristic absorption maximum of phenylalanine in GS. The total area of the GS peak as determined by Jasco ChromPass software was used as a measure of concentration. For calibration, commercial GS was used, and individual calibration curves were constructed for three concentration ranges: 10 to 100 mg/liter, 0.1 to 1 g/liter, and 1 to 10 g/liter.

### Table 1. Biosynthesis of GS by different strains of *A. miguulanus* and their phenotype composition

<table>
<thead>
<tr>
<th>Strain</th>
<th>GS production</th>
<th>Observed phenotype(s)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>In NBYS</td>
<td>In YP</td>
</tr>
<tr>
<td></td>
<td>Absolute (mg/liter)</td>
<td>Specific (mg/g DCW)</td>
</tr>
<tr>
<td>ATCC 9999T</td>
<td>150</td>
<td>50</td>
</tr>
<tr>
<td>DSM 2895T</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DSM 5668</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DSM 5759</td>
<td>100</td>
<td>50</td>
</tr>
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</table>

**Fig. 1.** Morphologically distinct colony variants coexist in *A. miguulanus* ATCC 9999T, which we distinguish as types I through VI.
Pure colony morphology phenotypes of *A. migulanus* ATCC 9999<sup>T</sup>. Each colony morphology variant was isolated and grown as a pure culture. For this purpose, a single characteristic 4-day-old colony was picked to obtain a spore suspension. The spores were then plated on LBY agar, and the resulting colonies were described in terms of their most distinct features (Table 2). The full phenotype typically became manifest after 1 week of cultivation (2 days at 37°C, followed by 5 days at room temperature). Among all developed phenotypes, only one (stemming from type V) corresponds to the morphology described for the original GS-producing phenotype R: rough, dull, dense, beige colonies 5 to 7 mm in diameter with pronounced rugose centers (Fig. 2A). As mentioned above, this variant was found only in *A. migulanus* ATCC 9999<sup>T</sup>. In DSM 2895<sup>T</sup>, instead of the expected R phenotypes, we observed only S forms. Notably, the shapes of these colonies deviate from that of the S phenotype described earlier (54). In the 5- to 7-mm colonies obtained from type IV (Fig. 2E), the convex profile was confined to a central region of 3 to 5 mm, while the large (10- to 12-mm) colonies from type VI (Fig. 2F) were not convex but planar, with a dense crater-like center. Therefore, we describe these two smooth morphology variants as SC and SP, respectively. We did not find either of the other previously described planar phenotypes, P<sup>+</sup> and P<sup>−</sup> (54), in the isolated dissociants; therefore, new names are introduced here. The rough, translucent, glistening, flat, and iridescent colonies de-

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Size (mm)</th>
<th>Color</th>
<th>Surface</th>
<th>Center properties</th>
<th>Biofilm formation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC of GS&lt;sup&gt;b&lt;/sup&gt; (mg/liter)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>5–6</td>
<td>Yellowish gray (beige)</td>
<td>Rough, dull, opaque</td>
<td>Dense, rugose, raised</td>
<td>2</td>
<td>400</td>
</tr>
<tr>
<td>RC</td>
<td>6–8</td>
<td>Yellowish gray (beige)</td>
<td>Partially rough, dull, opaque</td>
<td>Dense, convex</td>
<td>1</td>
<td>40</td>
</tr>
<tr>
<td>RP</td>
<td>7–9</td>
<td>Yellowish gray (beige)</td>
<td>Partially rough, dull, opaque</td>
<td>Dense, planar, with point elevation</td>
<td>4</td>
<td>40</td>
</tr>
<tr>
<td>RT</td>
<td>10–12</td>
<td>Yellowish gray (beige)</td>
<td>Rough, glistening, transparent</td>
<td>Diffuse</td>
<td>1–2</td>
<td>40</td>
</tr>
<tr>
<td>SC</td>
<td>10–12</td>
<td>Whitish, gray</td>
<td>Smooth, glistening, opaque</td>
<td>Dense, smooth, convex</td>
<td>4</td>
<td>80</td>
</tr>
<tr>
<td>SP</td>
<td>10–14</td>
<td>Whitish, gray</td>
<td>Smooth, glistening, opaque</td>
<td>Dense, smooth, planar crater-like</td>
<td>&lt;1</td>
<td>80</td>
</tr>
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</table>

<sup>a</sup> Upon growth in YP medium without agitation.  
<sup>b</sup> Resistance to external GS.

**FIG. 2.** Pure phenotypes of *A. migulanus*, identified as the R form derived from type V (A), the RC form derived from type I (B), the RP form derived from type II (C), the RT form derived from type III (D), the SC form derived from type IV (E), and the SP form derived from type VI (F). The panels on the left show the homogenous populations on LBY agar plates, and those on the right show individual colonies (magnification, ×7 to ×10).
veloped from type III are named RT (Fig. 2D), and we describe the pure colonies arising from type I as RC, as they possess a pronounced dull convex center (Fig. 2B). The phenotype from type II is named RP, as the center was planar with small point-like elevations (Fig. 2C). The colony morphology phenotypes designated RC and RP partially resemble the previously described P\textsuperscript{126}/H11001 phenotype. Notably, irrespective of their type, all dissociated colonies exhibit rough, glistening, transparent, and undulated irregular edges.

Growth and GS production of the different phenotypes of \textit{A. migulanus} ATCC 9999\textsuperscript{T}. Obviously, our most intriguing question concerned the ability of the different colony morphology phenotypes to produce the antimicrobial peptide GS. The productivity was examined in two cultivation media, which differ in AN content. First, a sporulation-promoting medium, NBYS, was used to search for a correlation between the sporulation peculiarities and the GS yield. Second, an AN-rich medium, YP, was used to assess the yield under conditions that are optimal for overproduction of GS (25). As seen from Fig. 3 and 4, the R phenotype showed the highest absolute (in grams per liter) and specific (in grams per gram of DCW) production of GS. The cultures were cultivated either in NBYS sporulation medium (A and B) or in YP medium, which is optimal for GS production (C and D). GS yields were determined at the times indicated by arrows in panels A and C.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{(A and C) Growth curves of phenotype variants of \textit{A. migulanus} ATCC 9999\textsuperscript{T}: R (filled circles), RT (open circles), RP (filled squares), RC (open squares), SC (filled triangles), and SP (open triangles). (B and D) Corresponding absolute (g/liter) and specific (g/g DCW) production of GS. The cultures were cultivated either in NBYS sporulation medium (A and B) or in YP medium, which is optimal for GS production (C and D). GS yields were determined at the times indicated by arrows in panels A and C.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4.png}
\caption{Bacterial growth (OD\textsubscript{660}) and absolute yield of GS, monitored as a function of time for the pure R phenotype of \textit{A. migulanus} ATCC 9999\textsuperscript{T} in YP medium.}
\end{figure}

with markedly lower yields. In contrast, colonies of the two smooth phenotypes (SC and SP) did not show any significant GS production even in YP medium. This finding explains the observed failure of DSM 2895\textsuperscript{T} and DSM 5668 to produce GS in terms of their colony morphology, since these strains are present as mixed SC/SP and pure SC phenotypes, respectively.

We also monitored the time-dependent growth of the different colony morphology phenotypes, to compare their kinetics and final cell densities. After 20 to 24 h of cultivation in NBYS medium (Fig. 3A), the R form had accumulated its maximum biomass and reached the highest density of all phenotypes (OD\textsubscript{660} = 12). The growth extent of RC, RP, and RT colonies was significantly less, reaching an OD\textsubscript{660} of only ~7. Both SC and SP variants grew to intermediate values (OD\textsubscript{660} ~ 10). Interestingly, in YP medium (Fig. 3C), the smooth phenotypes showed the lowest biomass accumulation (OD\textsubscript{660} ~ 8) and were the fastest to reach the stationary growth phase (20 h). Due to a delay in spore outgrowth (24, 27), the R variant exhibited a distinctly long lag phase (up to 16 h), and in contrast to all other variants the R form was still in the exponential phase even after 70 h of cultivation, reaching OD\textsubscript{660} values of 14. The RT, RP, and RC colonies showed an intermediate behavior, entering the stationary phase after 40 to 48 h. The production of GS by the colonies exhibiting rough phenotypes started in all cases after a certain cell density had been reached (OD\textsubscript{660} of 5 to 6). The time-dependent yield of GS in both media followed the shape of the growth curve, with a time shift of about 7 h (Fig. 4), and fully correlated with biomass accumulation. Remarkably, among the peptide-producing phenotypes, the R variant was exceptional in always showing the highest absolute GS production due to both high biomass accumulation and maximal specific GS production (Fig. 3).

Growth and GS production of the different phenotypes of \textit{A. migulanus} ATCC 9999\textsuperscript{T}. Obviously, our most intriguing question concerned the ability of the different colony morphology phenotypes to produce the antimicrobial peptide GS. The productivity was examined in two cultivation media, which differ in AN content. First, a sporulation-promoting medium, NBYS, was used to search for a correlation between the sporulation peculiarities and the GS yield. Second, an AN-rich medium, YP, was used to assess the yield under conditions that are optimal for overproduction of GS (25). As seen from Fig. 3 and 4, the R phenotype showed the highest absolute (in grams per liter) and specific (in grams per gram of DCW) productivity, irrespective of the cultivation medium. The other rough variants (RC, RP, and RT) were able to produce GS as well, but

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{FIG. 5. (A and C) Growth curves of phenotype variants of \textit{A. migulanus} ATCC 9999\textsuperscript{T}: R (filled circles), RT (open circles), RP (filled squares), RC (open squares), SC (filled triangles), and SP (open triangles). (B and D) Corresponding absolute (g/liter) and specific (g/g DCW) production of GS. The cultures were cultivated either in NBYS sporulation medium (A and B) or in YP medium, which is optimal for GS production (C and D). GS yields were determined at the times indicated by arrows in panels A and C.}
\end{figure}
different phenotypes must be attributed to variable regulation of gene expression.

**Resistance to external GS.** Since GS is an antimicrobial peptide that permeabilizes bacterial membranes, we were intrigued as to whether and how *A. migulanus* was able to evade such damage in its different phenotypes and stages of growth. The newly identified variants were therefore tested for their sensitivity to externally added GS. According to the MICs in Table 2, all of them were resistant to 40 to 80 mg/liter of GS, which is significantly higher than the usual MIC range for other gram-positive bacteria (<2 mg/liter [2, 7, 47]). Most fascinatingly, under the conditions tested we found that the R form is once again unique in being resistant to peptide concentrations as high as 400 mg/liter.

**Growth and spore formation.** For each variant, the spore formation process was examined by phase-contrast microscopy. The phenotypic features are summarized in Table 3 and Fig. 5, revealing some peculiarities of the vegetative cells and spore morphologies. The diameters of the vegetative cells of the R, SC, and SP phenotypes were 1 to 1.2 μm, while the RC, RP, and RT forms were somewhat slimmer (1.0 μm). The latter three variants had a characteristic cell length of 5 to 10 μm, and only the R-type cells were found to be 3 to 6 μm, being really “brevis,” which means “short.” Vegetative cells of the R and RP phenotypes were always highly mobile throughout cultivation, while RT, SC, and SP forms were visibly less mobile during early cultivation times. The cells of the RC variant were essentially immobile.

The spore-forming potential of the phenotypes in NBYS culture was assessed from the kinetics of total biomass accumulation on the one hand and from the absolute spore-forming activity of the individual vegetative cells on the other. All of the phenotypes investigated, except for R, started to sporulate after 16 h of growth in NBYS medium. In cultures of the R phenotype, the first spores were seen only after 20 h of growth, but after 28 h, most of the vegetative R cells already contained endospores. The sporulation frequency of all other variants was significantly lower even at longer cultivation times. Table 3 shows the number of spores that were obtained from the different colony morphology variants. The biomass production by the R variant was 1.5 to 2 times higher than that by the others. A comparison of the absolute spore forming activity (measured as spore-forming units per gram DCW) reveals that the disso-

ciants have a reduced ability to form spores.

The pure R form produced ellipsoidal endospores (1.5 by 2.5 μm) that were located subterminally in a distinctly swollen sporangium. In contrast, the other rough variants observed here (RC, RT, and RP) produced somewhat smaller, slender spores (0.8 to 1.0 by 1.2 to 1.5 μm) in an unswollen sporangium in a subterminal location. Interestingly, only the R form produced exclusively phase-bright spores, while the RC, RP, and RT spores were predominantly phase-bright with a few phase-dark spores as well (Fig. 5B). The SC and SP variants were highly heterogeneous in spore size and in the swelling properties of the sporangium. Also, an increased heterogeneity in the refractivity of the spores was characteristic for these variants. Differences in the release of spores from vegetative cells were assessed in 48-h cultures. A virtually complete release from the vegetative cells was observed only for the R form (Fig. 5A), whereas the cultures of the RC, RP, and RT variants contained a mixture of spores with sporulated and nonsporulated vegetative cells. The spores of the smooth variants remained in the sporangium (Fig. 5C and D). A palisade arrangement of the released cylindrical phase-bright spores of the RC variant from DSM 5759 and the SC variant of DSM 5668 was occasionally noted.

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**TABLE 3. Properties of vegetative cells and spores from the different colony morphology phenotypes of *A. migulanus***

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Vegetative cells</th>
<th>Sporangium</th>
<th>Spores</th>
<th>Sporulation activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Length (μm)</td>
<td>Motility</td>
<td>Size distribution</td>
<td>Refractivity (phase-contrast)</td>
</tr>
<tr>
<td>R</td>
<td>3–6</td>
<td>++ +</td>
<td>Swollen</td>
<td>Hom</td>
</tr>
<tr>
<td>RC</td>
<td>5–8</td>
<td>–</td>
<td>Unswollen</td>
<td>Het</td>
</tr>
<tr>
<td>RP</td>
<td>5–8</td>
<td>+</td>
<td>Unswollen</td>
<td>Het</td>
</tr>
<tr>
<td>RT</td>
<td>5–8</td>
<td>+</td>
<td>Unswollen</td>
<td>Het</td>
</tr>
<tr>
<td>SC</td>
<td>5–10</td>
<td>+</td>
<td>Swollen/unswollen</td>
<td>Het</td>
</tr>
<tr>
<td>SP</td>
<td>5–10</td>
<td>+</td>
<td>Swollen/unswollen</td>
<td>Het</td>
</tr>
</tbody>
</table>

*a* Motility was estimated in an NBYS culture grown for 16 h. ++ +, fast; +, slow; –, almost absent.

*b* Hom and Het, homogeneous and heterogeneous size distributions, respectively.

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**FIG. 5.** Representative spore suspensions of different phenotypes of *A. migulanus*, which were homogeneous in size and refractivity for variant R (A) but heterogeneous in morphology and refractivity for variants RC (B), SC (C), and SP (D).
DISCUSSION

When a bacterial phenotype is unstable, new colony morphologies can start to form, and this phenomenon is known as bacterial dissociation. It was noticed as early as 1921 (8) and was reviewed in detail by Braun (6). Dissociation had been initially attributed to spontaneous mutations and their subsequent selection. Later on, some underlying molecular mechanisms for phenotypic variations in bacteria were discovered. Namely, concerted alterations in the expression of one or more genes were shown to cause discrete dichotomous or multiphasic phenotype changes, which are described as phase or antigenic variations (6, 12, 17, 35, 42–44). These variations are random in the sense that it is impossible to predict which individual cell will undergo the switch, hence they concern the population as a whole. Phase variation is a reversible (ON/OFF) switch in the expression of certain genes, resulting in the appearance of two distinct subpopulations, which may also be accompanied by changes in the antigenic properties of the bacterium. These involve, for example, functional proteins integrated in the cell wall of gram-positive bacteria, such as transporters, porins, receptors, and enzymes. Alterations in these surface antigens lead to modifications in the cell wall or surface protein content and are directly reflected in a change of the colony morphology phenotype (4). In a previous study, the R, P⁺, P⁻ and S forms of B. brevis var. G.B. (=A. migulanus) were shown to exhibit different antigenic properties (26), thus representing antigenic variations. In being reversible (as shown above), they are also an example of phase variation.

It is important to emphasize the practical relevance of our observation that some of the A. migulanus phenotypes (SP and SC) have lost their ability to produce the antibiotic peptide GS. Therefore, to produce high yields of GS in YP medium, colonies of the R variant should preferably be picked, followed by the RP, RC and RT forms. By growing an inefficient GS producer in YP medium without agitation, where the bacteria are able to form a biofilm, it appears possible to regenerate the valuable R form. Since phenotypic variations are known to be modulated by environmental conditions, these may not have been ideal in the course of long-term preservation. Our analysis of the cultivation conditions in terms of GS yield and phenotype stability has thus led to practical recommendations for preserving the GS productivity of valuable strains. Temperature and medium composition seem to be the most important factors for cultivation. Initially, the GS producer was described as a thermotolerant strain, grown at temperatures up to 60°C (14). Later, a phenotypic dichotomy (dissociation into R and S phenotypes) was shown, and the optimal growth temperature for the GS-producing R form was determined to be 40 to 42°C, in contrast to 37°C for the unproductive S phenotype (14, 55). Furthermore, below 32°C even the R cultures completely stopped producing GS (39). The DSMZ and ATCC use 30°C and 37°C, respectively, to grow the A. migulanus strains. Temperature thus seems to be a strong selection factor that can lead to new phenotypes. Differences in the cultivation temperature may also explain the diversity in terms of the degree of dissociation of DSM 2895V versus ATCC 99997V.

Biosynthesis of the antimicrobial peptide GS was found to be preserved or even to increase when the bacteria were cultivated in medium with an elevated AN content. The original strain of B. brevis var. G.B. had been grown in yeast medium containing 10% yeast autolysate (AN = 500 mg/dl) and 0.5% glucose (14). LBS medium with 10 g/liter tryptone and 5 g/liter yeast extract Difco (AN = 70 to 80 mg/dl) was found to be suitable for both extensive sporulation and moderate GS production (27). The correlation between AN content and GS production was clearly illustrated by cultivating a GS producer on semisynthetic media with AN contents of 180, 225, and 310 mg/dl and by subsequently estimating the degree of antibiotic activity using a bioassay. The production of GS increased two-and fourfold, respectively, achieving an activity that is equivalent to a yield of up to 1.7 g/liter of GS (40). The highest reported yields of 2.5 g/liter GS were obtained in YP medium (25), where the actual AN levels (500 mg/dl) are as high as in the medium initially used by Gause and Brazhnikova (14).

Nutrient broth, which is recommended for A. migulanus in most culture collections, however, contains only ca. 25 mg/dl AN and is definitely not optimal for GS production. Therefore, to avoid or at least to reduce degenerative dissociation of the original producer, the strain should be grown under conditions optimal for GS synthesis: at a temperature of 40 to 42°C, and in a medium supplemented with at least 50 to 100 mg/dl AN (for instance, NBYS or LBY medium). Moreover, when the formation of a biofilm was promoted under such conditions (i.e., growth without agitation), we observed a spontaneous reversion even of the nonproducing phenotypes into the valuable GS-producing forms (Fig. 6).

Usually, spore-forming bacteria are stored in the form of spore suspensions in water. However, according to our experience, the spore suspensions of the R form did not maintain a stable phenotype any longer than half a year when stored at 4°C (data not shown). Longer storage led to elevated levels of dissociation into the RC, RP, and smooth variants. In contrast, we found that the spore suspensions preserved as 30% glycerol stocks at −20°C were stable at least for 3 years (data not shown). In view of the apparent stochastic nature of bacterial dissociation, we suggest that it is very important to examine any culture before use to determine whether it is still homogeneous. For this purpose, individual spores should be regularly replated on the agar medium, and the colony morphology should be checked for the presence of the R form. We have demonstrated here that certain colony features such as roughness (rough versus smooth), color (beige versus white), the ability to grow as a biofilm on the surface of nonagitated liquid media, the size of the vegetative cells (short versus long), and the size distribution of the spores (homogeneous versus heterogeneous) correlate with GS production (Tables 2 and 3 and Fig. 3). The homogeneous physical state of the spores from the R phenotype and the heterogeneous refractivity of the spore suspensions from all other variants can be used as additional markers of GS-producing activity. A large amount of phase-dark, rubbery spores in the spore-forming culture is a distinct feature of the GS-negative phenotypes SC and SP. The optimal time for harvesting the spores should be determined microscopically, as A. migulanus DSM 5759 (RC form) revealed more phase-dark spores when cultivated for longer than 3 days. It is therefore likely that the phase-dark spores of the nonproducing phenotypes had already started the germination process, still being present in the spore-forming culture. Such behavior of spores may be promoted by the absence of GS.

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Bacterial cells can also be stored in the dry state, and spore-forming bacteria can be directly preserved as dry spores. Both approaches may be successfully applied to the GS-producing strains of *A. migulanus*. However, we note that spores have to be obtained under optimal cultivation conditions (see above).

It has been reported that when R-form spores are lyophilized in sand and stored at room temperature, they retain their ability to produce GS for as long as 3 years (10). To preserve the viability and stability of GS production, lyophilization of the vegetative cells instead of spores has also been attempted (51). Interestingly, when cultivated under identical conditions, the lyophilized vegetative cells produce more biomass and correspondingly greater absolute amounts of GS than the lyophilized spores.

The correlation between bacterial dissociation, sporulation, resistance to GS, and GS production itself is also interesting from a molecular perspective. Since the peptide is synthesized nonribosomally, it cannot be directly generated from a single gene. Obviously, some structural or functional modifications of the proteins in the biosynthesis apparatus must be responsible for the phenomena reported here. The membrane localization of certain GS-synthesizing enzymes (28) and their possible involvement in the observed environmental adaptation raise questions concerning the biological role of GS in *A. migulanus*. Besides its apparent antibiotic effect against other bacterial species, the peptide is actively involved in regulating the dormant stage of the life cycle of *A. migulanus*. For example, GS eliminates a deficiency in the production of dipicolinic acid during spore formation (24). The peptide itself remains strongly associated with the endospore surface and enhances endospore stability and hydrophobicity (31).

In the outgrowing spores, GS interferes with the membrane functions, such as respiratory processes and transport of L-alanine (27, 33). Furthermore, GS appears to modulate the metabolism of the outgrowing spores by delaying RNA and protein synthesis (11, 33). It has been suggested that the absence of GS is directly responsible for spore defects, increased heat sensitivity, and an earlier outgrowth of the spores from GS-negative mutants (27, 33).

The increased amount of spores, which cannot leave the vegetative cells in GS-negative phenotypes (Fig. 5), observed here may reflect the need to recruit GS in the process of spore release. As we have demonstrated here, the various phenotypes of *A. migulanus* differ in their intrinsic resistance against externally added GS (Table 2). This finding suggests that changes in the colony phenotype are associated with either modifications in the cell envelope structure or modulations in the cell immunity mechanisms. Establishing the detailed molecular mechanisms and proteome expression profiles of the phenotypic variations of *A. migulanus* may help to explain the possible role of GS in the dynamic behavior of the bacterial populations and their interaction with the environment.

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References


