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# Analysis of Expression of the Alpha-Toxin Gene (*hla*) of *Staphylococcus aureus* by Using a Chromosomally Encoded *hla*::*lacZ* Gene Fusion

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**The staphylococcal alpha-toxin (Hla) is a major virulence factor contributing to *Staphylococcus aureus* pathogenesis. To elucidate the conditions influencing *hla* expression, the determinant was fused to *lacZ*, the reporter gene coding for β-galactosidase. The *hla*::*lacZ* fusion was integrated into the chromosome of the wild-type *S. aureus* strain Wood 46, leading to the variant Wood 46-3. Alpha-toxin expression was found to be dependent on temperature, showing a maximum at 42°C. Furthermore, the indicator strain showed a growth phase-dependent *hla* regulation which was influenced by temperature. At 37°C, induction of *hla*::*lacZ* expression occurred in the late exponential phase of growth, whereas at 42°C, a strong induction was observed as early as the mid-exponential phase. These observations were verified by Northern blot analysis of *hla* mRNA and by Western blot (immunoblot) analysis of culture supernatants of strain Wood 46. It was additionally found that the induction of *hla* transcription at 42°C was not coupled with higher concentrations of *agr* RNAIII, the effector molecule of the global regulator *agr*. Furthermore, expression of the alpha-toxin was repressed at a high osmolarity. It was also shown that oxygen is essential for *hla* expression and that cultivation of the *S. aureus* strain Wood 46-3 on solid medium and in the presence of carbon dioxide stimulated *hla* transcriptional activity.**

*Staphylococcus aureus* is an important pathogenic bacterium which causes a variety of human infectious diseases, including endocarditis, osteomyelitis, dermonecrosis, skin abscesses, and pneumonia (51). The organism produces a large number of extracellular and cell-associated proteins, many of which are involved in pathogenesis, such as alpha-toxin, toxic shock syndrome toxin (TSST), enterotoxins, protein A, and others (23). The alpha-toxin (Hla) is one of the major virulence factors of *S. aureus* and is produced by the majority of the strains (5, 25, 38, 40). It is a pore-forming toxin and has cytolytic, hemolytic, dermonecrotic, and lethal activities. A wide range of cell types, including erythrocytes, monocytes, lymphocytes, macrophages, epithelial cells, fibroblasts, and keratinocytes, is affected by alpha-toxin (4, 5, 49, 52).

Like most staphylococcal exoproteins, alpha-toxin is not expressed constitutively, but its production is regulated by an array of extracellular and intracellular signals. At least three global regulatory loci are involved in the regulation of alpha-toxin production. The best-investigated global regulatory system is the accessory gene regulator (*agr*), which contributes to the postexponential growth phase regulation of a number of unlinked genes (1, 36). In vitro, most exoproteins, including alpha-toxin, are preferentially produced in the postexponential growth phase, while cell surface proteins are downregulated in this growth phase (1, 26). Another global regulatory locus, designated staphylococcal accessory regulator (*sar*), has been shown to control the *agr*-mediated pathway (11, 12, 35). A third regulatory locus, called *xpr* (extracellular protein regulator), has recently been described and regulates exoproteins at the transcriptional level (48). However, it has also been speculated that additional factors may be involved in the regulation of alpha-toxin expression (50). Limited information about the conditions influencing alpha-toxin expression exists. Previous hemolysis studies revealed an influence of growth phase (14), glucose (15), carbon dioxide, and certain amino acids on alpha-toxin production (53).

To investigate the influence of environmental conditions on the expression of the alpha-toxin gene of *S. aureus* more precisely, a chromosomally encoded transcriptional *hla*::*lacZ* fusion was constructed and used in several assay systems.

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## MATERIALS AND METHODS

**Strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. *S. aureus* Wood 46 is an alpha-toxin-producing strain and was used to introduce a *hla*::*lacZ* gene fusion into the chromosome. RN4220 is a restriction-negative mutant of *S. aureus* 8325-4 and is capable of accepting *Escherichia coli* DNA (28). MC4100, a LacZ-negative *E. coli* strain (9), was used as the host strain in cloning experiments. pDH32M is a *Bacillus subtilis* integration vector, containing a promoterless *lacZ* gene preceded by the *B. subtilis spoVG* ribosome binding site (27). pBT1 (8) is a temperature-sensitive shuttle vector and was constructed by ligating a 2.3-kb *Pvu*II-*Bam*HI fragment from pBR322 with a 4.0-kb *Pst*I (filled in by Klenow fragment)-*Bam*HI fragment of pTV 1 (ts) (54).

**Media, chemicals, and enzymes.** *E. coli* strains were grown in LB broth (44). For DNA and RNA extractions as well as for exoprotein analysis, *S. aureus* strains were cultured in brain heart infusion (BHI) broth (Difco, Augsburg, Germany). For reporter gene studies, strain Wood 46-3 was cultivated in modified B broth consisting of 1% peptone (Roth, Karlsruhe, Germany), 0.5% yeast extract (BRL, Eggenstein, Germany), 0.5% NaCl (Roth), and 0.1% $K_2HPO_4$ (Merck, Darmstadt, Germany) if not stated differently. The recombinant *E. coli* and *S. aureus* clones were cultivated under selective antibiotic pressure with either ampicillin (100 μg/ml) or chloramphenicol (10 μg/ml), respectively. Antibiotics were purchased from Sigma, Deisenhofen, Germany. Restriction enzymes, Klenow fragment, and T4 DNA ligase were purchased from Pharmacia, Freiburg, Germany. For detection of $Lac^+$ colonies, LB plates containing 0.01% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; GERBU, Gaiberg, Germany) were used.

**Bacterial growth conditions.** *S. aureus* Wood 46-3 was cultivated by diluting an overnight culture 1:100 in 100-ml flasks that contained 20 ml of modified B broth.

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Description | Source or reference |
|---|---|---|
| Strains | | |
| *S. aureus* | | |
| Wood 46 | High-level production of alpha-toxin | 21 |
| Wood 46-3 | Derivative of Wood 46 with *hla*::*lacZ* fusion | This work |
| RN4220 | Derivative of 8325-4, efficient acceptor of *E. coli* DNA | 28 |
| *E. coli* MC4100 | F$^-$ *araD139* D(*argF lac*)*U196 rspL150 relA1 deoC1 ptsF25 rbsR flbB5301* | 9 |
| Plasmids | | |
| pDH32M | Integration plasmid for *B. subtilis* containing promoterless *lacZ*; Ap$^r$ Cm$^r$ | 27 |
| pBT1 | *S. aureus-E. coli* shuttle vector containing PE 194 (ts) and col E1 replicons; Ap$^r$ Cm$^r$ | 8 |
| pKO3 | pBT 1 containing promoterless *lacZ* gene; Ap$^r$ Cm$^r$ | This work |
| pKO8 | pDH32M containing transcriptional *hla*::*lacZ* fusion; Ap$^r$ Cm$^r$ | This work |
| pKO10 | pBT1 containing the *hla* promoter transcriptionally fused to *lacZ*; Ap$^r$ Cm$^r$ | This work |

Unless otherwise indicated, the cultivation was performed in a shaker at 180 rpm at 37°C. Samples were collected during the first 10 h at hourly intervals and after 14, 18, and 24 h to determine β-galactosidase activity and for measuring the optical density at 600 nm ($OD_{600}$). Strain Wood 46 was cultivated under the same conditions, and either cells were collected for RNA extraction or supernatants were used for Western blot (immunoblot) analysis. Temperature effects were studied at 30, 37, and 42°C. For experiments investigating the influence of osmolarity on alpha-toxin production, B broth was modified with respect to its NaCl, KCl, or sodium glutamate concentration, which ranged from 0 to 1.2 M. Solid cultures were grown as indicated in Results on B agar. Cultivation under anaerobic conditions was performed with tryptic soy broth and agar (Difco) by use of the anaerobic system Anaerocult (Merck). To investigate the effect of $CO_2$, B agar plates were incubated as indicated in Results in the presence of 5% $CO_2$.

**Recombinant DNA techniques.** Endonuclease restrictions, ligations, Klenow reactions, and electrophoresis were performed as recommended by the manufacturers. Isolation and purification of DNA fragments from agarose gels were performed with the Geneclean kit (Bio 101, Vista, Calif.). Isolation of plasmid DNA from *E. coli* was carried out by the alkaline method of Birnboim and Doly (6). The same procedure was modified by the addition of 50 μg of lysostaphin (Sigma) per ml in the lysis step to isolate plasmid DNA from *S. aureus*. Plasmid DNA was introduced into competent *E. coli* MC4100 by the $CaCl_2$ method (44). *S. aureus* was transformed by electroporation (45). All plasmid constructions were done with *E. coli* and subsequently transferred into the restriction-deficient *S. aureus* strain RN4220 by electroporation prior to transforming *S. aureus* Wood 46.

**Construction of plasmids.** As indicated in Fig. 1, the alpha-toxin promoter was amplified from *S. aureus* Wood 46 by PCR by use of the primers 5′-GTTTGA TATGGAATTCCTGAATTTTTC-3′ (1090 to 1063 nt upstream of the translation initiation signal), generating an *Eco*RI site at the beginning, and 3′-CTA CTTTTACTTTTGTGCATACCTAGGAGTCA-5′ (7 nt upstream to 26 nt downstream of the translation initiation signal), generating a *Bam*HI site at the end, and cloned into pDH32M, creating pKO8. A 4.6-kb *Eco*RI-*Nsi*I fragment of pKO8, containing the transcriptional fusion of the *hla* promoter and the promoterless *lacZ* gene, was cloned into the *Eco*RI-*Eco*RV restricted shuttle vector pBT1, generating pKO10. Plasmid pKO3 was constructed by cloning a 3.5-kb *Eco*RI-*Nsi*I fragment of pDH32M, containing the promoterless *lacZ* gene, into the *Eco*RI-*Eco*RV-digested shuttle vector pBT1 and used as a negative control in β-galactosidase assays.

**Construction of a single-copy *hla*::*lacZ* fusion.** The procedure to construct the transcriptional *hla*::*lacZ* fusion in strain Wood 46 is indicated in Fig. 1. The recombinant plasmid pKO10, carrying the *hla*::*lacZ* fusion, was transformed into *S. aureus* Wood 46. Transformants were grown overnight at 30°C in the presence of chloramphenicol to generate a population of plasmid-bearing cells. Serial dilutions of this culture were plated onto BHI agar plates with chloramphenicol and incubated at the nonpermissive temperature of 43°C. To determine whether pKO10 was integrated into the upstream sequence of the *hla* gene of strain Wood 46, chloramphenicol-resistant colonies at the nonpermissive temperature were analyzed by Southern hybridization.

**Southern blot analysis and generation of DNA probes.** Chromosomal DNA was prepared from cells grown overnight in BHI broth by a method described by Pospiech and Neumann (41). The protocol was modified by replacing lysozyme with 50 μg of lysostaphin per ml. Following its digestion with restriction endonucleases, the resulting fragments were separated by electrophoresis with a 0.8% agarose gel. The transfer of the DNA onto a Biodyne B membrane (Pall, Portsmouth, United Kingdom) was performed as described previously (2) with a vacuum blot apparatus (Vacu Gene; Pharmacia, Freiburg, Germany). For the detection of the *hla* gene, the cloned 1.1-kb *Eco*RI-*Bam*HI fragment containing the *hla* promoter was used as a DNA probe. The hybridization was carried out with an ECL direct nucleic acid labelling and detection system from Amersham (Braunschweig, Germany) as described in the manufacturer's instructions.

**Extraction of RNA and DNA-RNA hybridization.** RNA was prepared by use of the RNeasy system of Qiagen, Hillen, Germany. After electrophoresis of samples with the same amount of total cellular RNA, determined by measuring the $A_{260}$, the gel was analyzed by Northern blot hybridization as described previously (2). The probes, a 722-nt intragenic *Cla*I fragment (21) from the *hla* gene and a 837-nt *Ssp*I fragment specific for *agr* RNAIII (26), were labelled by use of an ECL kit from Amersham, and the hybridization was performed as described in the manufacturer's instructions. The signals were quantified by densitometric scanning.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli with discontinuous 12.5% acrylamide gels (30). For immunoblot analysis, proteins were transferred onto a nylon membrane by semi-dry electroblotting in a graphite chamber by the method described by Kyhse-Anderson (29). Following blotting of the membranes, blocking was done with 5% nonfat dry milk (Bio-Rad, München, Germany) in Tris-buffered saline for 1 h. The filters were then incubated for 1 h with a polyclonal anti-alpha-toxin antibody in Tris-buffered saline containing 0.05% Tween 20 (Sigma) following a 0.5-h incubation with horseradish peroxidase-conjugated antirabbit antiserum (DAKO, Hamburg, Germany) diluted 1:1,000. Blots were developed with ECL substrate (Amersham), and signals were quantified by densitometric scanning.

**β-Galactosidase tests.** β-Galactosidase assays were performed with a Galacto Light Plus chemiluminescent reporter assay system (Tropix, Bedford, Mass.). After cultivation as described above, bacterial cells were harvested, washed, and resuspended in 0.9% NaCl. The cell suspension was adjusted to an $OD_{600}$ of 1.0. A 0.5-ml volume of this suspension was centrifuged (8,000 × *g*, 10 min), and the cell pellet was resuspended in lysis buffer (0.01 M potassium phosphate buffer [pH 7.8], 0.015 M EDTA, 1% Triton X-100, 50 μg of lysostaphin per ml) and incubated at 37°C for 10 min. Following centrifugation (10 min, 10,000 × *g*), 10 μl of the culture supernatant was used in a β-galactosidase assay as described in the manufacturer's instructions. The β-galactosidase activity was measured with a LB 9501 luminometer (Berthold, Wildbad, Germany) with a 300-μl automatic injector and a 5-s integral.

**Statistics.** Mean values ± standard deviations were calculated by the method described by Cavalli-Sforza (10).

## RESULTS

**Construction of the chromosomally encoded *hla*::*lacZ* fusion in *S. aureus* Wood 46.** The recombinant plasmid pKO10, carrying the *hla*::*lacZ* fusion, was transformed into *S. aureus* Wood 46, and integration occurred after a temperature shift as described in Materials and Methods. To determine whether pKO10 was integrated into the upstream sequence of the *hla* gene of strain Wood 46, chloramphenicol-resistant colonies at the nonpermissive temperature were analyzed by Southern hybridization with the cloned *hla* fragment used as a probe. As demonstrated in Fig. 2, the wild-type strain Wood 46 hybridized with both a single *Eco*RI fragment (5.5 kb in size) and a *Hin*dIII fragment (4.8 kb in size) of the chromosomal DNA. The appearance of two *Eco*RI fragments of 14.1 and 2.2 kb and two *Hin*dIII fragments of 13.9 and 1.7 kb indicated a site-specific integration of pKO10 into the promoter region of the

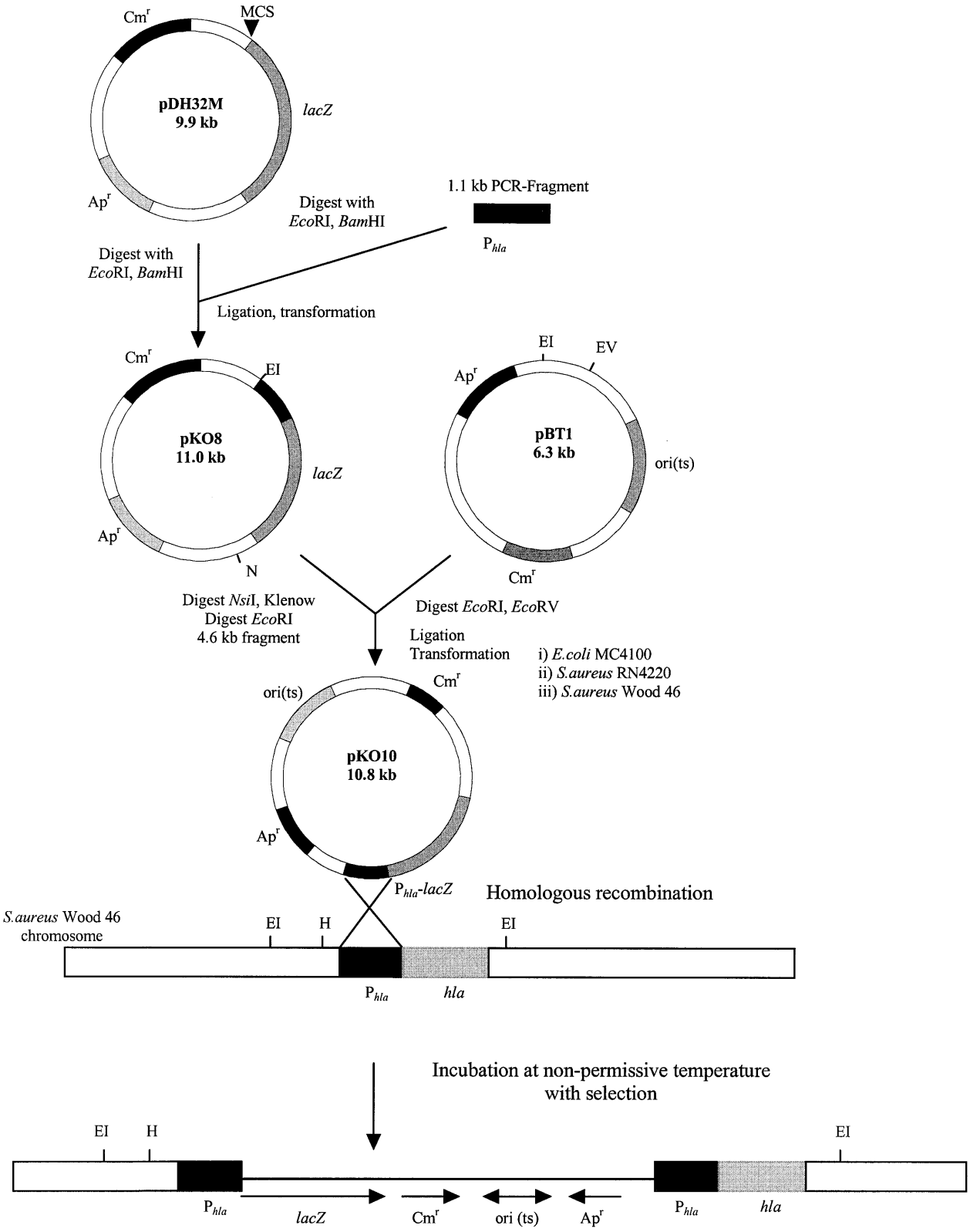


FIG. 1. Strategy for the construction of the chromosomally encoded *hla*::*lacZ* fusion in *S. aureus*. The *hla* promoter region used to generate the transcriptional fusion was amplified by PCR. *Eco*RI and *Bam*HI restriction sites were introduced to facilitate subsequent cloning. Plasmid pKO10 was first transformed into *E. coli* MC4100 cells following transformation of *S. aureus* RN4220 and *S. aureus* Wood 46 cells. The homologous recombination was carried out as described in the text. Ampicillin resistance ($Ap^r$) is expressed only in gram-negative species, whereas chloramphenicol resistance ($Cm^r$) is expressed in gram-positive species. Ori (ts) is a gram-positive temperature-sensitive origin of replication from pTV 1 (ts). Restriction site abbreviations: B, *Bam*HI; EI, *Eco*RI; EV, *Eco*RV; N, *Nsi*I. MCS, multiple-cloning site.

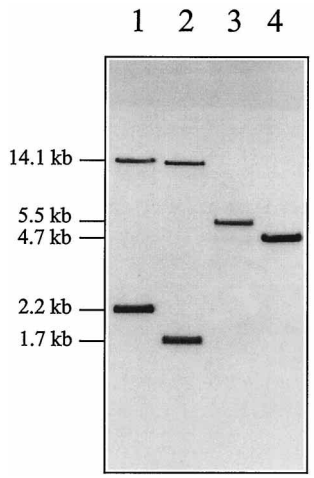


FIG. 2. Southern blot analysis of chromosomal DNA from *S. aureus* Wood 46-3 (lanes 1 and 2) containing a *hla*::*lacZ* fusion and *S. aureus* wild-type strain Wood 46 (lanes 3 and 4). Chromosomal DNA was restricted with *Eco*RI (lanes 1 and 3) and *Hin*dIII (lanes 2 and 4) and hybridized with a peroxidase-labeled 1.1-kb *Eco*RI-*Bam*HI fragment containing the *hla* promoter region.

*hla* gene of Wood 46. Since the promoter upstream of the *hla* open reading frame remained active, Wood 46-3 is a Hla-positive strain.

**Influence of temperature and growth phase on *hla*::*lacZ* expression.** The expression of the *hla*::*lacZ* fusion in *S. aureus* Wood 46-3 was examined at 30, 37, and 42°C. The highest level of β-galactosidase production, representing *hla* expression, was obtained at 42°C (Fig. 3). In addition, at 42°C, a difference in initial β-galactosidase production levels was observed. While a strong induction of *hla* expression occurred at the end of the exponential phase at 37°C, a high β-galactosidase activity was detected as early as the mid-log phase at 42°C. Cultures at both temperatures showed comparable growth rates (Fig. 3). At 30°C, Wood 46-3 grew more slowly than it did at 37 or 42°C and β-galactosidase activity was detected over a period of 36 h. The β-galactosidase activity first became detectable after 6 h, and maximal expression occurred after 22 h, reaching one-third of the maximal *hla*::*lacZ* activity of cultures grown at 37°C (data not shown).

**Influence of temperature and growth phase on *hla* mRNA production.** To determine if β-galactosidase activity of the *hla*::*lacZ* fusion in strain Wood 46-3 reflected *hla* promoter activity, the *hla* mRNA production of the wild-type strain Wood 46 was determined by Northern blot analysis of total cellular RNA and quantified by densitometric scanning. Samples were prepared from cells grown at either 37 or 42°C after 2, 3, 4, 6, and 10 h of incubation. As shown in Fig. 4, the kinetics of *hla* mRNA levels in Wood 46 were at both temperatures similar to those determined by analyzing *hla* expression with the *hla*::*lacZ* transcriptional fusion. There was no *hla* transcript detectable after 2 h of cultivation at 37°C, but low levels of *hla* mRNA were produced at 42°C. After 3 h, a strong induction of *hla* mRNA expression was detected at 42°C, while at 37°C only a weak *hla* mRNA-specific signal emerged. However, after 4 h, *hla* expression increased strongly at 37°C, while the concentration of *hla* mRNA was only slightly increased at 42°C. The highest level of *hla*-specific mRNA was detected after 6 h at both 37 and 42°C. After 10 h, the *hla*-specific mRNA began to decrease slightly. At every time during the growth cycle, the level of *hla* mRNA at 37°C was lower than that at 42°C. Both cultures exhibited comparable growth rates (data not shown).

**Influence of temperature on alpha-toxin production.** To determine the effect of temperature on alpha-toxin production, Wood 46 cells were cultivated in liquid culture and samples were collected from the supernatant throughout the whole growth period. Western blot analysis revealed that the alpha-

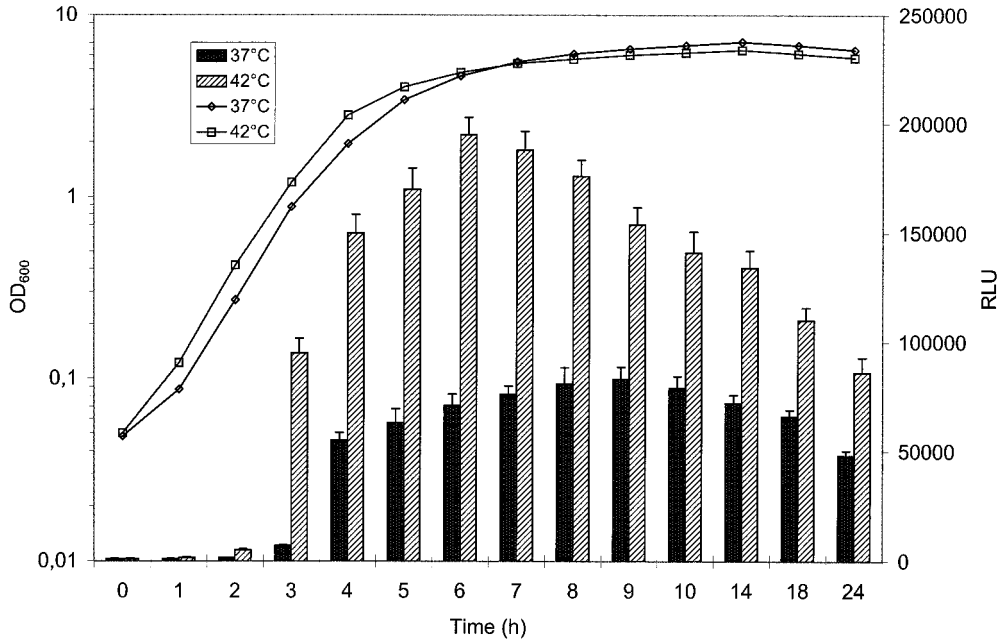


FIG. 3. Kinetics of transcription of a chromosomal *hla*::*lacZ* fusion at 37 and 42°C. β-Galactosidase activity indicated by bars is expressed in relative light units (RLU). Assays were performed as described in Materials and Methods. Mean values ± standard deviations of four experiments are given. Lines indicate representative growth as measured by $OD_{600}$. Growth experiments were repeated four times.

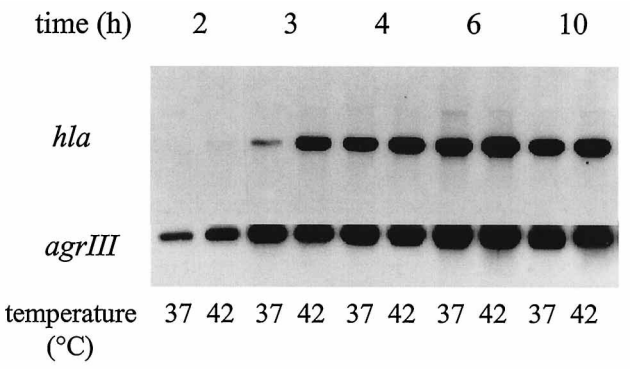


FIG. 4. Northern blot analysis of total RNA from *S. aureus* Wood 46 cultured at 37 and 42°C. The blot shows representative results of one of three experiments. RNA was prepared from cells after 2, 3, 4, 6, and 10 h of growth in B medium. Six micrograms of RNA was loaded into each well. After electrophoresis and blotting, the filter was first probed for *hla* mRNA and afterwards for *agr* RNAIII.

toxin concentration in the supernatant was higher following cultivation of Wood 46 at 42°C than at 37°C (Fig. 5). After 4 h, 14-fold more alpha-toxin was detected by densitometric scanning in supernatants from cultures grown at 42°C than in those grown at 37°C. Although during the following period of growth the alpha-toxin concentration increased at both temperatures, the ratio between the levels of alpha-toxin produced at 42 and 37°C decreased. After 10 h, only twofold more alpha-toxin was detected in cultures grown at 42°C than after growth at 37°C.

**Influence of temperature and growth phase on *agr* mRNA production.** The alpha-toxin gene is subject to *agr*-mediated regulation (26). To determine whether the effect of temperature on the level of *hla* expression was due to changes in *agr* mRNA levels, the concentration of *agr* RNAIII, the effector of *agr* regulation, was determined in Northern blot experiments and quantified by densitometric scanning. As shown in Fig. 4, *agrIII* expression started in the early exponential phase, and after 2 h, twofold more RNAIII was detected following cultivation of Wood 46 cells at 42 than at 37°C. However, during the following 4 h of growth, no differences in RNAIII concentrations were determined between cells grown at either 37 or 42°C. During the stationary phase, RNAIII levels were slightly increased in cells cultivated at 42°C compared to those grown at 37°C. This indicates that induction of *hla* expression during the mid-log phase and higher *hla* mRNA levels at 42°C

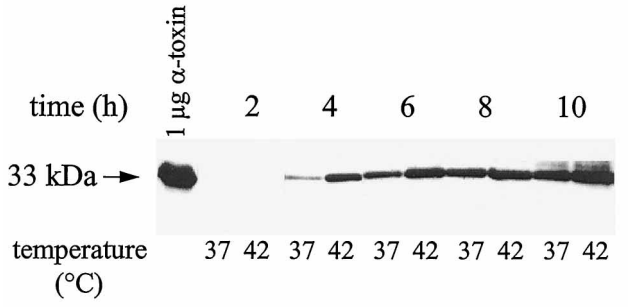


FIG. 5. Immunoblot analysis of alpha-toxin production during the growth of strain Wood 46 at 37 and 42°C. The blot shows representative results of one of three experiments. Culture supernatant samples were taken following 2, 4, 6, 8, and 10 h of incubation, and 10 μl of each sample was loaded onto the gel. As a control, 1 μg of purified alpha-toxin (Sigma) was used in lane 1.

throughout the whole growth cycle are not strictly correlated with higher *agr* RNAIII concentrations.

**Influence of osmolarity on *hla*::*lacZ* expression.** To examine the influence of osmolarity on *hla* expression, strain Wood 46-3 was grown in B broth containing sodium chloride at concentrations ranging from 0 to 1.2 M. The cultures were monitored with regard to β-galactosidase activity until the early stationary phase. The maximal expression at every osmolarity is indicated in Fig. 6. The highest level of β-galactosidase activity was detected after cultivation in B medium lacking NaCl. Higher osmolarities decreased the expression of the *hla*::*lacZ* fusion. While moderate concentrations ranging from 0.1 to 0.4 M NaCl diminished β-galactosidase activity twofold, 0.8 M NaCl decreased the *hla*-dependent β-galactosidase production fivefold, and almost no *hla*::*lacZ* activity was detected at 1.2 M. To determine if the expression of *hla* was dependent on osmolarity rather than sodium ion concentration, similar experiments were performed with potassium chloride and sodium glutamate as the osmolyte. As shown in Fig. 6, the *hla*::*lacZ* fusion strain expressed similar levels of β-galactosidase activity at lower KCl concentrations, but at higher osmolarities, the repression of β-galactosidase activity was more pronounced; however, the growth rate was slightly higher in medium with high concentrations of KCl than in medium with high NaCl levels (Fig. 7A and B). Sodium glutamate also affected *hla*::*lacZ* expression (Fig. 6). However, strong repression occurred even at moderate osmolyte levels (0.1 to 0.4 M). Higher osmolyte concentrations led to a strong repression of β-galactosidase activity. The growth kinetics were roughly identical for cultures grown with equal osmolarities of NaCl or sodium glutamate, respectively (Fig. 7A and C).

**Influence of media, oxygen, and carbon dioxide on *hla*::*lacZ* expression.** The influence of growth on solid medium on *hla*::*lacZ* expression was investigated. Therefore, strain Wood 46-3 was grown over a period of 48 h on B agar, and every 4 h, a sample was taken to detect β-galactosidase activity. As shown in Fig. 8A, *hla*::*lacZ* expression was slightly higher in solid than in liquid cultures. To examine the influence of oxygen on *hla* expression, cultures of Wood 46-3 were grown anaerobically on solid medium as well as in liquid cultures. β-Galactosidase production was monitored over a time period of 48 h. β-Galactosidase activity was not detected upon cultivation in liquid cultures or on solid medium (Fig. 8B). Although *S. aureus* is a facultative anaerobic bacterium, the growth rate of strain Wood 46-3 under anaerobic conditions was drastically reduced, reaching an $OD_{600}$ of 2.3, in comparison with an $OD_{600}$ of 7.8 after aerobic growth. Additionally, the influence of carbon dioxide on *hla*::*lacZ* expression upon cultivation on solid medium was investigated over a period of 36 h. An almost twofold increase of β-galactosidase production was detected at a concentration of 5% $CO_2$ (Fig. 8C), although the growth rate of strain Wood 46-3 was slightly reduced in the presence of 5% $CO_2$ (visible observation).

## DISCUSSION

The production of bacterial virulence factors is frequently influenced by various environmental stimuli (31). In this study, the environmental regulation of the *hla* gene of *S. aureus*, coding for the alpha-toxin, a major virulence factor involved in *S. aureus* pathogenesis, was investigated. Therefore, a transcriptional *hla*::*lacZ* fusion was constructed and integrated into the chromosome of *S. aureus* Wood 46. In consequence, β-galactosidase production is equivalent to *hla* mRNA production and could be quantified as an indicator of the *hla* promoter activity. We showed that β-galactosidase activity of

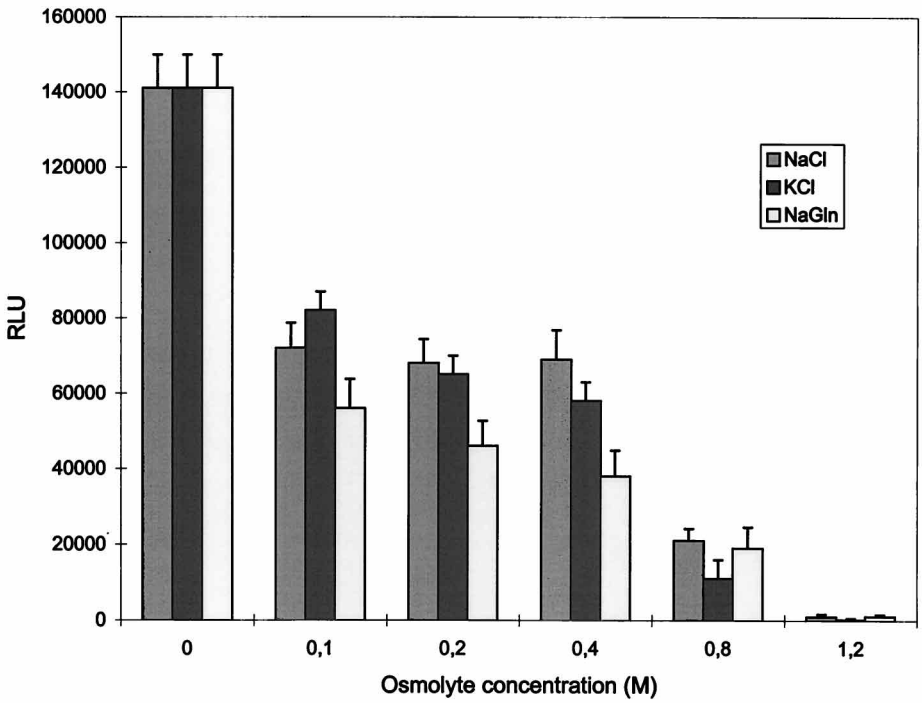


FIG. 6. β-Galactosidase production at varying osmolarities given as relative light units (RLU). Cultures were grown as described with various concentrations of NaCl, KCl, and sodium glutamate. β-Galactosidase production was monitored until the cells reached the early stationary phase. Maximal β-galactosidase activity is indicated. Data are means ± standard deviations of three experiments.

strain Wood 46-3 harboring the *hla*::*lacZ* fusion was equivalent to the *hla* mRNA concentration in Wood 46 determined by Northern blot experiments. Thus, our system was proven to be suitable to monitor *hla* expression, and it could be used to investigate the influence of environmental signals on the expression of the alpha-toxin gene. To our knowledge, this is the first report on a chromosomally integrated gene fusion of a staphylococcal virulence determinant and a reporter gene in *S. aureus*. Compared with a described transcriptional *hla*::*blaZ* fusion on a multicopy plasmid (36), the wild-type gene fusion has the advantage that undesirable multicopy effects which could counteract the regulatory events under question in plasmid systems is excluded. By use of a very sensitive assay to detect β-galactosidase activity, poor β-galactosidase values obtained by the classical method by Miller (33) were overcome. The system could also be applied to the investigation of transcriptional regulation of other target genes.

Previous investigations showed that alpha-toxin expression is affected by growth phase (36), glucose, and pH (43). In this investigation, several new findings are presented: (i) expression of *hla* is temperature regulated, showing an optimum at 42°C; (ii) growth phase-dependent induction of *hla* expression at 42°C occurred as early as the mid-log phase and was not correlated with increased *agr* RNAIII levels; (iii) *hla* expression was osmotically regulated, showing a strong repression at high osmolarities; (iv) oxygen was essential for *hla* expression; and (v) carbon dioxide and solid media enhanced *hla* promoter activity.

Temperature is known as an environmental factor governing the expression of virulence genes in various bacterial pathogens (31). The increase of the temperature to 37°C was shown to be a signal for human pathogens to enter their host from the environment. Therefore, many virulence genes and regulators are optimally expressed at 37°C, including *E. coli* P (19) and S fimbriae (46), the virulence regulator *virR* of *Shigella flexneri* (22), and the *Bordetella pertussis bvg* regulon (42). Others are preferentially expressed at a high level at a low temperature, e.g., flagella of *Legionella pneumophila* (39), the cholera toxin of *Vibrio cholerae* (16), or *Yersinia enterocolitica* enterotoxin *Yst* (32). In contrast, alpha-toxin expression was enhanced at 42°C. Interestingly, TSST-1, another staphylococcal exotoxin and a member of the pyrogenic exotoxins, is also optimally expressed at high temperatures (24). In this context, it is noteworthy that staphylococcal alpha-toxin itself triggers the production of interleukin 1 and tumor necrosis factor alpha, two potent endogenous pyrogenics (3, 5). High-temperature induction of alpha-toxin production is not limited to strain Wood 46. Preliminary studies with clinical *S. aureus* isolates showed similar results (37).

Growth phase-dependent regulation of exoprotein synthesis in *S. aureus*, including alpha-toxin production, has previously been investigated and is controlled by the global regulatory locus *agr* (1, 26, 36). For alpha-toxin, however, it has also been shown that an additional factor emerging at the end of the exponential phase is necessary for full activation of *hla* expression (50). Our results were consistent with these observations. In addition, data presented in this study indicate that regulation by growth phase can be altered by an effect of high temperature. At 42°C, induction of *hla* expression occurred in the mid-log rather than the late log phase of growth, and at this time, there was no difference in the concentration of RNAIII in cells grown at either 37 or 42°C. This indicates that the earlier induction of *hla* expression at 42°C is not mediated by *agr*. Timing and level of *hla* expression may be affected by additional factors. Moreover, a strict correlation between *hla* message and protein levels was not observed. It has been shown that RNAIII activates *hla* gene expression at both transcriptional and translational levels (34, 36). The translational

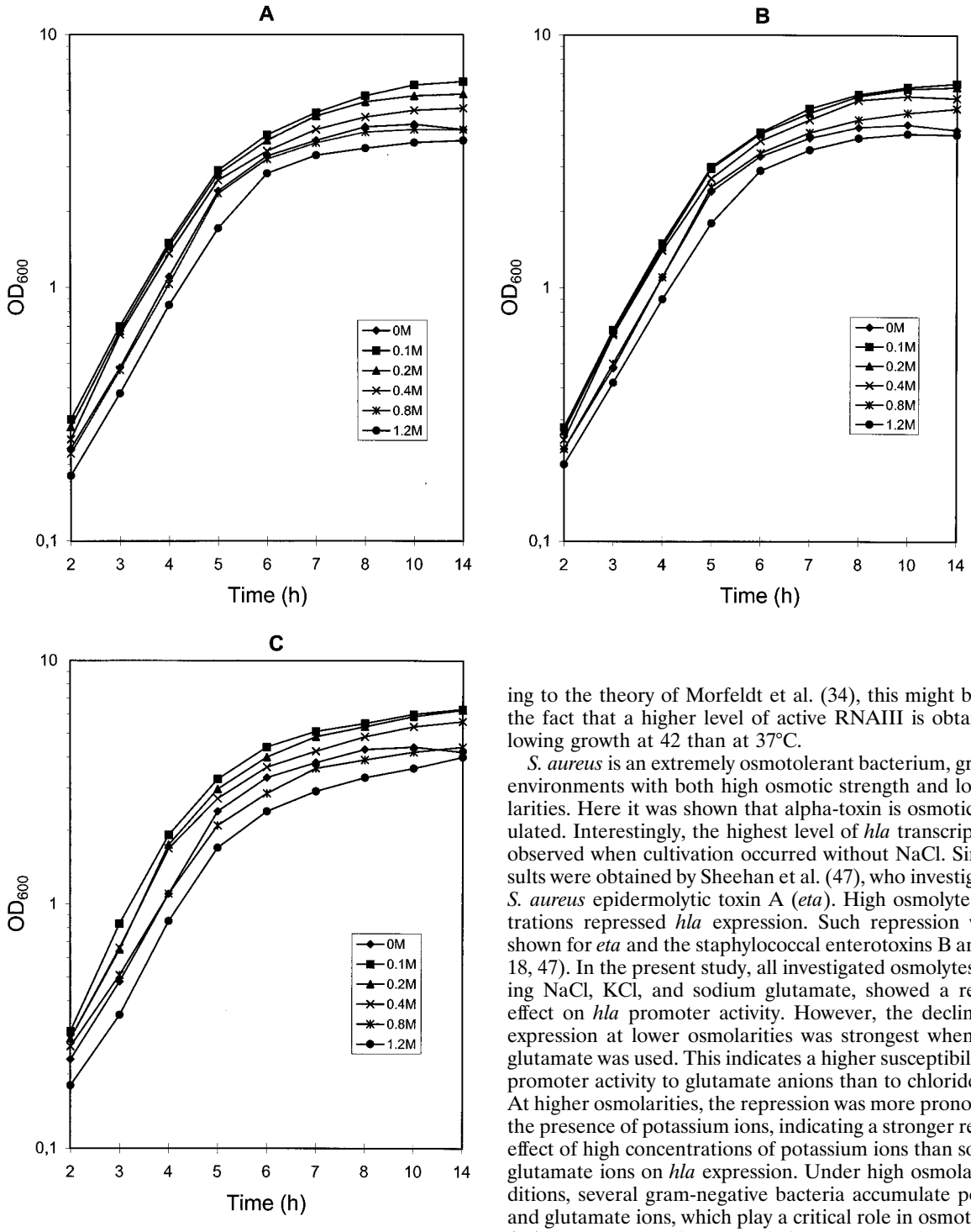


FIG. 7. Growth kinetics of strain Wood 46-3 following culture under different osmolarity conditions. Growth occurred in the presence of either sodium chloride (A), potassium chloride (B), or sodium glutamate (C). Lines indicate representative growth as measured by $OD_{600}$. Growth experiments were repeated three times.

activation is achieved by a direct interaction of RNAIII with *hla* mRNA (34). It has also been speculated that RNAIII can switch from an active to an inactive form and vice versa by interaction with an accessory factor (34). Our data suggest a higher efficiency of translation of *hla* mRNA at 42°C. According to the theory of Morfeldt et al. (34), this might be due to the fact that a higher level of active RNAIII is obtained following growth at 42 than at 37°C.

*S. aureus* is an extremely osmotolerant bacterium, growing in environments with both high osmotic strength and low osmolarities. Here it was shown that alpha-toxin is osmotically regulated. Interestingly, the highest level of *hla* transcription was observed when cultivation occurred without NaCl. Similar results were obtained by Sheehan et al. (47), who investigated the *S. aureus* epidermolytic toxin A (*eta*). High osmolyte concentrations repressed *hla* expression. Such repression was also shown for *eta* and the staphylococcal enterotoxins B and C (17, 18, 47). In the present study, all investigated osmolytes, including NaCl, KCl, and sodium glutamate, showed a repressive effect on *hla* promoter activity. However, the decline of *hla* expression at lower osmolarities was strongest when sodium glutamate was used. This indicates a higher susceptibility of *hla* promoter activity to glutamate anions than to chloride anions. At higher osmolarities, the repression was more pronounced in the presence of potassium ions, indicating a stronger repressive effect of high concentrations of potassium ions than sodium or glutamate ions on *hla* expression. Under high osmolarity conditions, several gram-negative bacteria accumulate potassium and glutamate ions, which play a critical role in osmotolerance (13). However, such accumulation was not observed for *S. aureus* (20). It may therefore be concluded that the signal for osmolarity-induced repression of *hla* expression is not based on the intracellular concentration of potassium or glutamate ions. For *V. cholera* cholera toxin (16) and *E. coli* S fimbriae (46), optimal gene expression was reported at NaCl concentrations below 0.1 M and a strong gene repression occurred even at moderate osmolyte concentrations. The relative high *hla* promoter activity at moderate osmolyte concentrations could reflect a specific adaption of *S. aureus* to NaCl, which may be a common component of the habitat of this bacterium.

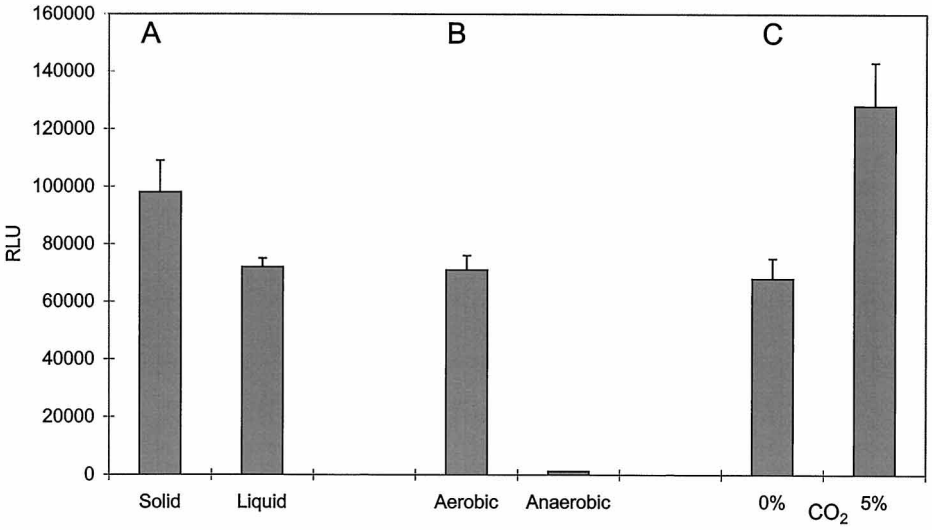


FIG. 8. β-Galactosidase production, given as relative light units (RLU), by the fusion strain Wood 46-3. (A) Growth on either solid or liquid medium; (B) growth under either aerobic or anaerobic conditions on solid medium; (C) growth without or with 5% $CO_2$ on solid medium. Data are means ± standard deviations of three experiments.

Early hemolysis studies revealed a critical role of oxygen in alpha-toxin production. Moreover, it was speculated that carbon dioxide enhances alpha-toxin production (53). The data presented in this study demonstrated an effect of oxygen and carbon dioxide on regulation of alpha-toxin production on transcriptional level. While oxygen was essential for *hla* expression, carbon dioxide enhanced *hla* promoter activity. It is interesting to note that oxygen is also essential for expression of TSST-1 of *S. aureus* and that $CO_2$ also has a stimulatory effect on TSST-1 production (24) and the enterotoxin production by *V. cholerae* (16). Moreover, the expression of *hla* was slightly induced after cultivation on solid medium. *S. aureus* is able to colonize a variety of tissues, and it has been shown that alpha-toxin causes necrosis in animal models (7).

The exact role of alpha-toxin in the manifestation of human diseases caused by *S. aureus* is presently unclear, and care must be taken when projecting in vitro data to the pathogenesis of *S. aureus* infections. However, environmental signals are potent stimuli governing expression of virulence factors. The results presented in this study revealed that alpha-toxin expression is altered by certain environmental stimuli which may contribute to the in vivo virulence of *S. aureus.*

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