Differential Expression of the Lactose Transporter Gene Affects Growth of *Staphylococcus aureus* in Milk

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ABSTRACT

In this study, differential display polymerase chain reaction (PCR) was used to search for unique or enhanced expression of genes in prevalent bovine mastitis-causing *Staphylococcus aureus* strains. Comparison of a pair of prevalent and rare strains revealed the differential expression of several genes. The lactose-specific permease, enzyme II (EII), was highly expressed in the prevalent strain. This gene was selected for further study due to its potential influence on bacterial growth, because lactose is the primary carbohydrate in milk. Growth analysis illustrated that prevalent strains reach significantly higher growth densities sooner than rare strains. Quantitative competitive reverse transcription PCR (QC RT-PCR) revealed increased EII mRNA expression in prevalent strains as compared to rare strains. Mutation of the EII gene resulted in abrogated growth and decreased EII mRNA expression in media containing lactose. These data suggest that increased EII expression may facilitate the pathogenesis of *S. aureus* mastitis by enhancing growth. This study is the first to implicate EII as a potential virulence factor in mastitis, and therefore may be useful in the development of novel therapeutic strategies against *S. aureus* mastitis.

(**Key words:** lactose transporter, *Staphylococcus aureus*, mastitis)

Abbreviation key: *agr* = accessory gene regulator, **BLAST** = Basic Local Alignment Search Tool, **CcpA** = catabolite control protein, **Cm** = chloramphenicol, **dNTP** = deoxynucleotide triphosphate, **EII** = enzyme II, **GAPDH** = glyceraldehyde-3-phosphate dehydrogenase, ***lac*** = lactose operon, **MMLV-RT** = moloney murine leukemia virus reverse-transcriptase, **ORF** = open reading frame, **PTS** = phosphotransferase system, **QC RT-PCR** = quantitative-competitive reverse-trans-

criptase PCR, **TSA** = tryptic soy agar, **TSB** = tryptic soy broth, **TSST-1** = toxic shock syndrome toxin-1.

INTRODUCTION

Strain typing has become a powerful tool for investigating the epidemiology of bacterial infections. *Staphylococcus aureus* strains have been subtyped by a wide variety of methods including: phage typing, ribotyping, plasmid analysis, pulse-field gel electrophoresis of genomic DNA fragments, multilocus enzyme electrophoresis, random amplified polymorphic DNA, and coagulase gene typing (Slanetz and Bartley, 1962; Baumgartner et al., 1984; Thomson-Carter et al., 1989; Musser et al., 1990; Goh et al., 1992; Saulnier et al., 1993; Matthews, 1994). Our laboratory utilized restriction fragment length polymorphism to subtype *S. aureus* strains obtained from clinical cases of mastitis from various countries, based on the coagulase gene (Aarestrup et al., 1995; Su et al., 1999). It was determined that only a few genotypes abound from each geographical location tested (Su et al., 1999). Further experiments showed that these predominant genotypes were significantly more resistant to neutrophil killing than rare genotypes (Su et al., 1999; Mullarky et al., 2001). Consequently, it was hypothesized that these prevalent strains may differentially express certain genes that better enable them to overcome essential host defense mechanisms and successfully colonize mammary tissue. Therefore, we utilized differential display to identify potential virulence factors produced by these prevalent *S. aureus* strains. Differential display was originally developed by Liang and Pardee to identify differentially expressed genes among two or more eukaryotic cell populations (Liang and Pardee, 1992). Since then, differential display has been successfully employed to identify candidate virulence factors in bacteria (Rindi et al., 1999; Achour et al., 2002). The ability to identify novel virulence genes would greatly improve our understanding of mastitis virulence.

Mastitis infection by *S. aureus* is a complex process involving many bacterial factors (Sutra and Poutrel, 1994). Bacterial growth is one such factor, and is sig-

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nificant in the virulence of mastitis-causing *S. aureus* (Chesbro et al., 1968; White et al., 1980; Odierno et al., 1994). While the mechanism by which growth affects virulence is not yet fully understood, recent studies are beginning to elucidate its role. One study recently demonstrated that growth is required for the production of the superantigen, toxic shock syndrome toxin-1 (TSST-1) (Timmins and Holland, 1999). They found that as *S. aureus* shifts down in growth rate during entry into the stationary phase of growth, TSST-1 increases considerably. Furthermore, growth is known to play a role in the regulation of virulence factors through the accessory gene regulator (*agr*) (Novick et al., 1993). *Staphylococcus aureus* is able to sense its growth density through the interaction of a quorum sensing mechanism produced by the *agr* locus (Peng et al., 1988). Consequently, *S. aureus* is able to express surface-associated products during early exponential phase and extracellular factors during postexponential phase (Novick et al., 1993). Therefore, any contribution to growth is likely to have a direct effect on virulence.

To metabolize carbohydrates and grow, many bacteria utilize the phosphoenol pyruvate-dependent phosphotransferase system (PTS), which couples translocation to phosphorylation of the transported carbohydrates (Postma et al., 1993; Lengeler et al., 1994). The *S. aureus* lactose-specific enzyme EII of the PTS, encoded by *lacE*, selectively transports lactose across the bacterial membrane (Reizer et al., 1988). EIII (*lacF*) phosphorylates lactose before it is cleaved into glucose and galactose-6-phosphate by phospho- β -galactosidase (*lacG*) (Hengstenberg et al., 1970). Lactose and galactose have been shown to induce the lactose operon (*lac*), although the common byproduct of their catabolism, galactose-6-phosphate, is the actual intracellular inducer (Morse et al., 1968). The *lac* operon is also subject to catabolite repression by the *lac* repressor, thus its expression is based on carbohydrate source (Oskouian and Stewart, 1990). Because EII plays such an important role in lactose metabolism, we hypothesized that the differential expression of EII can affect growth of *S. aureus* in a lactose-rich microenvironment, such as milk. Because growth has long been implicated in the virulence of *S. aureus* mastitis, influencing growth could have a significant impact (Chesbro et al., 1968; White et al., 1980; Odierno et al., 1994). To date, no studies have investigated the impact of EII expression on growth as it relates to mastitis. Understanding the relationship between carbohydrate metabolism and virulence may lead to the development of novel prevention strategies.

MATERIALS AND METHODS

Bacterial Strains

Before this study, a collection of 453 *S. aureus* isolates were obtained from samples of bovine mastitic milk,

which were acquired from a variety of geographical locations (the Czech Republic, France, Korea, and the United States). These isolates were subtyped into 40 types by restriction fragment length polymorphism, using the coagulase gene. Only a few genotypes predominated in each country (Su et al., 1999). *Staphylococcus aureus* strains used in the present study were considered prevalent if their genotype represented 40% or more of the isolates from one country. Conversely, strains were considered rare if their genotypes represented less than 10% of the isolates from one country. From this collection, a prevalent (A4) and rare strain (L42) were used for differential display PCR. For growth curves and EII mRNA expression, a total of 18 *S. aureus* strains were selected from both prevalent ($n = 9$) and rare ($n = 9$) genotypes. Prevalent strain, CZ8, was later selected for mutagenesis. All isolates were cultured in trypticase soy agar (TSA) with 5% sheep blood (BiMed, St. Paul, MN), and stored at -70°C in trypticase soy broth (TSB) with 15% glycerol until needed.

RNA Preparation and Differential Display PCR

Total RNA was collected from *S. aureus* strains and purified using Trizol reagent according to manufacturer's instructions (Life Technologies, Baltimore, MD). The total RNA samples were then treated with RNase-free DNase to eliminate DNA contamination. To enrich mRNA concentration, rRNA was subtracted from total RNA samples as described previously (Su and Sordillo, 1998). The pair of *S. aureus* strains used for differential display was grown in UHT milk for 5 h at 37°C , and the mRNA was isolated. The arbitrary primers used in this study are listed in Table 1. A total of 30 pairs of primers were designed for differential display PCR. The first primer of each set was used as a primer for reverse transcription to generate cDNA from mRNA (Table 1). To generate cDNA for PCR, 200 ng of mRNA was first denatured at 70°C and reverse transcribed into cDNA at 42°C for 1 h. The reaction mixture contained $1 \times$ reverse transcription buffer, $0.25 \mu\text{M}$ of an arbitrary primer, 2.5 mM of each deoxynucleotide triphosphate (dNTP), 40 U of ribonuclease inhibitor, and 200 U of moloney murine leukemia virus reverse transcriptase (MMLV-RT). After reverse transcription, mRNA was digested by ribonuclease H. The PCR was performed in a volume of $25 \mu\text{l}$ with $1 \times$ PCR buffer, $0.4 \mu\text{M}$ of the primer used for reverse transcription, $0.4 \mu\text{M}$ of the second arbitrary primer, $2 \mu\text{l}$ of reverse transcription products, 2.5 mM of MgCl_2 , 0.4 mM of each dNTP, $0.5 \mu\text{Ci}$ of ^{32}P dCTP, and 1 U of Taq DNA Polymerase (Promega, Madison, WI). The cycling conditions were 94°C (1 min), 30°C (2 min), 72°C (2.5 min) for 40 cycles.

Table 1. Differential display arbitrary primers.

Primer ID	Sequences
DP1	TGCCTAATAATGCTAAAC
DP2	AGGGCGTGGTGCAGGGCGGT
DP3	TCGGGGATGGGAGTGCTAATG
DP4	TGGCTATGTGCTATGGGTCTTT
DP5	ATGAAAAATGCTGGGCTTGGAGA
DP6	CCACGCGTGTGTGTGAAA
DP7	GAGGGCGGTGGCAGGAGTTAGG
DP8	AGGCCGTGTACTCCGTGATGG
DP9	GCTAAGGAGGCAGAGGGTGA
DP11	GGCCGAGGGTGTGAGGTTT
DP16	TCGGGGATGGGAGTGCTA
DP17	ATGAGGAAGACCAGGAAGAAGC
DP18	AAATGCTGAATAATCCGC
DP22	TACAGCATCATCTTTGTGCTCG
DP23	ATCTTGATGTCCACACAGAT

The following 30 primer sets were used for differential display PCR: DP1/DP6, DP1/DP8, DP2/DP3, DP2/DP7, DP3/DPI, DP3/DP5, DP3/DP16, DP4/DP2, DP4/DP11, DP4/DP8, DP4/DP9, DP5/DP1, DP5/DP2, DP5/DP8, DP5/DP9, DP6/DP9, DP7/DP4, DP7/DP5, DP7/DP17, DP8/DP1, DP8/DP4, DP8/DP5, DP8/DP9, DP8/DP18, DP9/DP2, DP9/DP11, DP22/DP2, DP22/DP4, DP23/DP1, DP23/DP2.

The PCR products were separated on a 6% polyacrylamide gel. After gel electrophoresis, the gel was dried and exposed to X-ray film at -70°C . Differentially amplified PCR products existing in all RNA preparations from three separate differential displays were then reamplified by PCR using the corresponding primers. The cycling conditions were 94°C (1 min), 60°C (1 min), and 72°C (1.5 min) for 30 cycles. The reamplified PCR products were cloned and used directly to generate probes for Northern blot analysis.

Northern Blot Analysis of Selected Differentially Expressed Genes

To confirm the findings in differential display PCR, Northern blot analysis was performed. Total RNA (10 μg) was electrophoresed in formaldehyde gels (1.2%). The RNA was then transferred to a Nytran membrane and cross-linked with UV radiation. DNA probes were labeled with ^{32}P (Amersham Pharmacia Biotech, Piscataway, NJ) using Prime-a-Gene Labeling System (Promega, Madison, WI). After hybridization at 68°C for 2 h, the membrane was washed and X-ray film exposed at -70°C . After confirmation by Northern blot analysis, differentially expressed genes were sequenced by cycle sequencing (Protein and DNA Chemistry Services, Biotechnology Institute and Bioprocessing Resource Center, 210 Wartik lab, University Park, PA). To determine the identity of the DNA sequences, Basic Local Alignment Search Tool (BLAST) search was conducted against the GenBank database (Altschul et al., 1990).

QC RT-PCR of EII mRNA

The forward and reverse EII primers were: (5'-CAGGAACGACAGCGAAATCTT-3') and (5'-GCTGTTGCGAATGACTAAATCTAA-3'). The EII internal standard primers using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were: ISfor (5'-TAATACGACTCACTA TAGGCGAGGAACGACAGCGAAATCTTGCATGGCCTTCCGTGTCC-3'), and ISrev (5'-TTTTTTTTTTTTTTT TGTCTGTTGCGAATGACTAAATCTAACAGCCCCA GCGTCAAAGGTG-3'). *Staphylococcus aureus* RNA (1 μg) was added to a RT mixture containing the following: 1 μg of 10^8 molecules of competing RNA internal standard (GAPDH), $5 \times$ MMLV buffer, 10 mM dNTP, 25 mM MgCl_2 , 10 U of MMLV-RT, 150 μg of oligo dT, and 8 U of RNasin. Each sample was incubated for 45 min at 37°C and 5 min at 95°C . Five microliters of the RT mixture was then added to a PCR mixture containing the following: $10 \times$ NEB buffer, 25 mM MgCl_2 , 10 pmol of forward and reverse EII primers, and 1 U of Taq polymerase. Thermal cycling parameters were 95°C (5 min) for 1 cycle; 94°C (10 s), 60°C (30 s), 72°C (45 s) for 30 cycles; and 72°C (5 min) for 1 cycle. RT-PCR products were then electrophoresed on 1.8% agarose gels and detected in the presence of ethidium bromide under UV illumination.

Growth Analysis

Overnight cultures of *S. aureus* strains were adjusted to an A_{540} of 0.01 and inoculated into either UHT milk or TSB containing the following changes: a) 136 mM lactose, b) 13.89 mM lactose, c) 13.89 mM glucose, or d) 13.89 mM lactose plus 13.89 mM glucose. Bacterial cultures were grown for 12 h at 37°C while shaking at 150 rpm. Aliquots taken every hour were serially diluted and plated on TSA.

EII Mutagenesis

The *S. aureus lacE* gene was interrupted using the chloramphenicol (Cm) resistance gene. The *lacE*, a 2040-bp fragment containing the entire *lacE* sequence was PCR amplified, digested with *Bam*HI, and ligated into pUC18: EI1K01 (5'-GTTGGGATCCCAGGGGATGCACGTTCTAAGT-3') and EI1K02 (5'-TTTCGGATCCATACGTATCCCATGCTACGCGACC-3'). The Cm gene was PCR amplified from pACYC184 (New England Biolabs, Beverly, MA) using primers containing *Spe*I sites: CM1 (5'-TGGCACTAGTGAGACGTTGATCGGCACGTA-3') and CM2 (5'-TAACACTAGTGCCCTGAACCGACGACCGGG-3'). The Cm fragment was digested and ligated into the *Spe*I site of the cloned *lacE* fragment. The *lacE::Cm* fragment was then ligated into *Bam*HI site of pCL52.1, and transformed into *E. coli*

DH5 α while selecting for with spectinomycin (50 μ g/ml) (Lin et al., 1994). The resulting plasmid was electroporated into *S. aureus* strain, RN4220 (Kreiwirth et al., 1983), selected for with tetracycline (20 μ g/ml) and incubated at 30°C. The *lacE* mutant construct was finally transformed into prevalent strain, CZ8, via electroporation. Allelic exchange proceeded as previously described (Lin et al., 1994). Tetracycline-sensitive colonies were grown at 37°C and screened by PCR, using primers: EIIKOTEST1 (5'-TGGAGAACACGCAGATA AAA-3') and EIIKOTEST2 (5'-TAGGAATGGGTAGTA AATAATAA-3'). Mutations were confirmed by PCR. Phospho- β -galactosidase assays were performed as previously described by Oskouian (Oskouian and Stewart, 1987), to determine whether the mutation in EII disrupted lactose transport.

Statistical Analysis

Significant differences between growth rates of prevalent and rare strains were determined with one-way ANOVAs using the Minitab (State College, PA) statistical program. The Student's two-sample *t*-test was used to determine significant differences among EII transcripts and specific time points during the growth analyses. Statistical significance was set as $P < 0.05$.

RESULTS

Differential Display Identifies EII as Having a Potential Effect on Growth in Milk

A total of 30 pairs of arbitrary primers were tested in this study (Table 1). Based on the fact that *S. aureus* has a genomic DNA of 2.8 Mbp, and assuming the average gene size is 1500 bp, there would be approximately 2000 genes available. Assuming each pair of primers can generate 10 PCR products and there are no significant overlaps among all PCR products, a total of 200 to 250 pairs of arbitrary primers would be needed to exhaust the screening for all possible *S. aureus* genes. Therefore, the results described in this paper accounts for approximately 15% of the *S. aureus* genome. Four pairs of these primers were successful at generating PCR products, while the other 26 pairs of primers produced 5 to 15 DNA bands. Northern blot analysis of 19 selected PCR products confirmed that eight clones were uniquely or highly expressed in the predominant strain A4, and one clone was highly expressed in the rare strain L42 (data not shown). A BLAST search demonstrated that nine positive clones had matches within the *S. aureus* genomic DNA sequence database. The cDNA sequence from clone UH-12 contained an open reading frame (ORF) that had a 93% similarity to the *S. aureus* Fibronectin-binding protein B amino acid se-

Table 2. The clones differentially expressed in predominant and rare *S. aureus* strains (A4 vs. L42).

ID	BLAST Search Results (GenBank)
UH-1/2	678 bp, 99% (627/643) identity to <i>Staphylococcus aureus</i> lactose-specific transporter enzyme II DNA sequence.
UH-12	421 bp, 93% (319/341) identity to <i>S. aureus</i> FnBP-B DNA sequence.
UH-4/2	532 bp, 94% (468/494) identity to a <i>S. aureus</i> hypothetical protein DNA sequence.
UH-19	342 bp, 97% (285/291) identity to toxic anion resistance protein homologue DNA sequence in <i>S. aureus</i> .
UH-6/1	422 bp, 96% (369/383) identity <i>S. aureus</i> aspartate-semialdehyde dehydrogenase DNA sequence.
UH-3/6	493 bp, 97% (451/464) identity to a <i>S. aureus</i> hypothetical protein DNA sequence.
UH-2	594 bp, 95% (552/579) identity to a <i>S. aureus</i> putative serine-threonine rich antigen sequence.
IH-4/7	338 bp, 98% (296/301) identity to <i>S. aureus</i> inositol-monophosphate dehydrogenase DNA sequence.
LIH-13	716 bp, 98% (698/707) identity to a <i>S. aureus</i> hypothetical protein DNA sequence.

quence. A different clone, UH-1/2, contained an ORF that had a 99% similarity to the *S. aureus* lactose-specific transporter EII amino acid sequence. The remaining clones included ORF that closely matched other *S. aureus* genes (Table 2).

To obtain a quantitative measure on whether EII was differentially expressed in milk, three prevalent and three rare strains known to have differentially expressed genes, were analyzed using QC RT-PCR. As shown in Figure 1, the prevalent strains produced significantly more EII transcripts than the rare strains,

Figure 1. Enzyme II (EII) mRNA expression between prevalent (■) and rare (□) *Staphylococcus aureus* strains grown in UHT milk. Data are expressed as means + standard error for three strains per group in duplicate assays. *Indicate significant differences between groups ($P < 0.05$).

confirming the results obtained from differential display PCR and Northern blot. Because EII is the lactose transporter in *S. aureus*, its expression may affect growth in an environment rich in lactose, such as milk. Therefore, we tested the growth of a total of 18 stains (nine prevalent and nine rare) in UHT milk over 12 h while growing at 37°C. The data in Figure 2 show that prevalent strains grew better than rare strains in UHT milk, reaching significantly higher densities in late-exponential and stationary phases, although growth rates remained unchanged. Based on these data, we hypothesized that increased EII mRNA expression is responsible for improved growth in a lactose-rich environment.

Correlation Between EII mRNA Expression and Growth

To determine how carbohydrate source affects EII mRNA expression, we conducted QC RT-PCR on 18 strains (nine prevalent and nine rare). Because the concentration of lactose is known to vary significantly in milk (Kitchen, 1981), it was important to use a medium in which the carbohydrate source could be controlled. Thus, TSB was modified to contain 13.89 mM glucose, 13.89 mM glucose and 13.89 mM lactose, 13.89 mM lactose, or 136 mM lactose. We chose a concentration of 136 mM lactose to simulate milk, since it contains 136 mM lactose on average (Kitchen, 1981). In addition, 13.89 mM lactose was chosen to determine whether a lower lactose concentration would affect EII mRNA expression, and to match the concentration of glucose found in stock TSB. QC RT-PCR revealed that prevalent strains produce significantly more EII transcripts when grown in media containing 13.89 mM and 136

Figure 2. Growth curve of prevalent (◆) and rare (■) *Staphylococcus aureus* strains grown in UHT milk. Each data point represents the mean $\pm$ standard errors for nine strains per prevalence group in duplicate assays. *Indicate significant differences between groups per time point ($P < 0.05$).

Figure 3. Enzyme II (EII) mRNA expression between prevalent (■) and rare (□) *Staphylococcus aureus* strains grown in tryptic soy broth containing 13.89 mM glucose, 13.89 mM glucose and 13.89 mM lactose, 13.89 mM lactose, or 136 mM lactose. Data are expressed as means $\pm$ standard error for nine strains per group in duplicate assays. *Indicate significant differences between prevalent and rare *S. aureus* strains ($P < 0.05$).^{a,b,c} Indicate significant differences with respect to culture media ($P < 0.05$).

mM lactose (Figure 3), demonstrating that lactose affects EII transcription. No significant differences in EII transcript production were observed between prevalent and rare strains grown in media containing both glucose and lactose, or glucose alone. Figure 3 also reveals a dose response in EII mRNA expression with respect to the percentage of lactose in the media, in both prevalent and rare strains. By increasing lactose concentration, EII transcript production also increases. Furthermore, both prevalent and rare strains produce significantly more EII transcripts when grown in 136 mM lactose, than when grown in 13.89 mM lactose with no glucose present.

To determine whether EII mRNA expression correlates with growth, the 18 isolates also were analyzed for their ability to grow in media containing different concentrations of glucose and lactose. When the strains were grown in media containing glucose, only minor differences appeared (Figure 4), indicating that both populations could utilize glucose efficiently. Conversely, prevalent strains displayed improved growth compared to rare strains during growth in media containing only lactose (Figure 4). Growth in 136 mM lactose reveals that rare strains require approximately an additional hour of growth during late-exponential phase to attain similar densities achieved by prevalent strains. The ability of prevalent strains to begin a shift-down in growth rate earlier may contribute to their virulence by increasing toxin production (e.g., TSST-1) before rare strains (Timmins and Holland, 1999).

Figure 4. Growth comparison of prevalent (■) and rare (□) *Staphylococcus aureus* strains grown in tryptic soy broth containing 13.89 mM glucose, 13.89 mM glucose and 13.89 mM lactose, 13.89 mM lactose, or 136 mM lactose after 8 h. Each data point represents the mean $\pm$ standard error for nine strains per prevalence group in duplicate assays. *Indicate significant differences between prevalent and rare *S. aureus* strains per time point ($P < 0.05$).

Impact of EII Mutagenesis on Growth

To establish whether EII expression directly affects growth, EII of the prevalent strain, CZ8, was mutated by inserting an antibiotic gene through allelic exchange, and verified by PCR. Mutagenesis of EII resulted in significantly lower expression of EII transcripts compared with wild-type (Figure 5), demonstrating

Figure 5. Enzyme II (EII) mRNA expression among prevalent (■), EII mutant (striped bar), and rare (□) *Staphylococcus aureus* grown in tryptic soy broth containing either 13.89 mM glucose or 136 mM lactose. Data are expressed as means $\pm$ standard error in triplicate assays.^{a,b} Indicate significant differences between *S. aureus* strains ($P < 0.05$). *Indicate significant differences with respect to growth media ($P < 0.05$).

Figure 6. Growth curve of prevalent (◆), Enzyme II (EII) mutant (▲), and rare (■) *Staphylococcus aureus* grown in UHT milk. Each data point represents the mean $\pm$ standard error of quadruplicate assays. *Indicate significant differences between prevalent and mutant *S. aureus* per time point ($P < 0.05$).

that the mutation was successful in altering expression of the EII gene. The mutant also displayed abrogated growth in media containing lactose, confirming that EII mRNA expression alters growth. In UHT milk, the mutant attained significantly lower growth densities than the wild-type prevalent strain (Figure 6). Conversely, few differences were observed between wild-type and mutant when grown in media containing glucose (Figure 7), demonstrating that the EII mutation is specific to growth in lactose. As with UHT milk, the mutant did not grow as well as the wild type in 136 mM

Figure 7. Colony-forming units of prevalent (■), Enzyme II (EII) mutant (striped bar), and rare (□) *Staphylococcus aureus* grown in tryptic soy broth containing 13.89 mM glucose or 136 mM lactose after 8 h. Each data point represents the mean $\pm$ standard error of quadruplicate assays. *Indicate significant differences between prevalent and mutant *S. aureus* per time point ($P < 0.05$).

lactose (Figure 7). Finally, the EII mutant displayed significantly reduced phospho- β -galactosidase activity, further demonstrating that the mutation in EII has a direct effect on lactose (Figure 8).

DISCUSSION

In this study, we were able to identify differentially expressed genes among bovine mastitis-causing *S. aureus* strains using differential display PCR. The screening presented in this paper is representative of approximately 15% of the *S. aureus* genome. Thus, further analysis of the *S. aureus* genome would likely identify additional differentially expressed genes than those presented in this report. Nevertheless, the identification of Fibronectin-binding protein B as a virulence factor in this experiment proved that differential display PCR could be a valuable tool to study other potential *S. aureus* virulence factors, as strong evidence exists to suggest that Fibronectin-binding protein B is a relevant virulence factor in *S. aureus* mastitis (Mamo et al., 1994; Sutra and Poutrel, 1994; Mamo et al., 1995; Park et al., 1999). The gene for the lactose-specific permease EII, *lacE*, also was identified as a differentially expressed gene. We chose to focus on EII, because it is responsible for active transport of lactose in *S. aureus*, which is the primary carbohydrate in milk (Postma et al., 1993). To our knowledge, this is the first report to suggest an association between EII expression and mastitis by affecting the growth of *S. aureus* in a lactose-rich environment, such as milk.

The results from this study show that EII mRNA expression is regulated by carbohydrate source. Transcription of the *lac* operon, and consequently EII, is controlled by the catabolite repressor and *lac* repressor (Oskouian and Stewart, 1987). In the presence of glucose, the catabolite repressor prevents transcription by binding to the promoter site of the *lac* operon (Oskouian and Stewart, 1990), thus corroborating the observed decrease in EII mRNA expression in the presence of glucose. Furthermore, *lac* repressor binding of the *lac* promoter region is inhibited in the presence of galactose-6-phosphate, a byproduct of lactose catabolism (Morse et al., 1968). Hence, the higher the concentration of lactose, the less likely the *lac* repressor will be able to prevent transcription of the *lac* genes. This theory is substantiated by our observations of a dose response in EII mRNA expression to an increase in lactose concentration.

In this study, we found that EII affects growth when *S. aureus* is cultured in UHT milk and media rich in lactose. While we found that growth rates were similar between prevalent and rare strains, prevalent strains appeared to enter stationary phase sooner. The ability

Figure 8. Activity of phospho- β -galactosidase by prevalent (■), Enzyme II (EII) mutant (striped bar), and rare (□) *Staphylococcus aureus* grown in tryptic soy broth containing 136 mM lactose. Units of phospho- β -galactosidase were measured using *O*-nitrophenol β -D-galactopyranoside in bacteria grown to mid-exponential phase. The data represent means + standard errors of three independent experiments. ^{a,b,c} Indicate significant differences between groups ($P < 0.05$).

to enter stationary phase earlier may contribute to virulence by allowing the bacteria to devote more energy into virulence factor production rather than growth. For example, production of known virulence factors such as α -toxin and TSST-1 do not reach maximum levels until *S. aureus* exits exponential growth (Coleman and Abbas-Ali, 1977; Timmins and Holland, 1999). Prevalent *S. aureus* strains achieved significantly higher growth densities than rare strains in UHT milk. Increased growth correlated with EII expression in milk, but the contribution of other milk components besides lactose cannot be overlooked. The mutation in EII was not enough to reduce the growth of the mutated prevalent strain to that of the selected rare strain (Figure 7), suggesting that other components contribute to the growth of these *S. aureus* strains in milk. For instance, one study found that certain enterotoxin-producing strains of *S. aureus* grew better in milk fat (cream) than others (Halpin-Dohnalek and Marth, 1989). Moreover, the compositional changes brought about during mastitis influence how *S. aureus* grows, since heme components and casein degradation play a role in improved *S. aureus* growth (Mattila et al., 1984; Kaartinen and Sandholm, 1987). Therefore, EII is likely to be one of many factors that can influence growth in milk.

The EII effect on growth in milk is confounded by the fact that lactose concentration is known to vary (Kitchen, 1981). For this reason, TSB was useful because the carbohydrate concentration can be better controlled. Growth in this media resulted in curves distinct from UHT milk, possessing less pronounced differences.

Nevertheless, prevalent strains appeared to grow better than rare strains in media containing lactose as the only carbohydrate. Interestingly, growth in media that contained glucose resulted in the fewest differences, suggesting to us that EII was responsible for the observed differences between prevalent and rare strains. Additionally, the mutation in EII abrogated growth in media containing only lactose, but not in media containing glucose, further substantiating that EII expression affects growth.

To elucidate whether EII expression is directly responsible for altering growth, we disrupted the *lacE* gene in a prevalent *S. aureus* strain. Mutagenesis of *lacE* resulted in lower growth densities compared with the wild-type strain when grown in lactose. Consistent with our previous observations, the *lacE* mutant grew as well as wild-type *S. aureus* in media containing glucose. These results confirm that the disruption was specific for lactose metabolism, and that the lactose-specific EII does not play a role in glucose metabolism in *S. aureus*, as it relates to growth. Disruption of *lacE* also resulted in decreased expression of EII transcripts and phospho- β -galactosidase activity. Taken together, these data confirm that growth is linked to EII expression in lactose. Without the lactose-specific EII to transport lactose across the bacterial membrane, PTS uptake of the carbohydrate would be prevented (Postma et al., 1993). In the absence of intracellular lactose, galactose-6-phosphate would not be generated. Consequently, transcription of the *lac* operon would not be initiated (Morse et al., 1968). Furthermore, the mutant was able to reach higher densities than the selected rare strain, suggesting to us that this rare strain may have deficiencies in other carbon metabolisms beyond that of lactose metabolism.

Because the *lac* operon regulates itself through a repressor and promoter, it is possible that a variation in the concentration of promoter sites, or repressor molecules, could result in variation in the remaining *lac* operon (Oskouian and Stewart, 1990). For example, increasing the concentration of *lac* promoter transcripts could titrate out the lactose repressor molecule, resulting in constitutive expression of the *lac* genes (Oskouian and Stewart, 1990). Thus, the results observed in this study may be due to differential expression of the repressor or promoter rather than just EII. Additionally, many gram-positive bacteria contain a global catabolite control protein (**CcpA**), which could play a role in the differential expression of the *lac* operon between prevalent and rare strains (Stewart, 1993; Henkin, 1996; Saier, 1996; Saier et al., 1996). However, the role of CcpA has yet to be defined and characterized in *S. aureus*. Regardless, EII remains valuable, as it is the only *lac* component to be surface-

associated and responsible for transporting lactose into the cell from its surroundings (Breidt et al., 1987; Kowolik and Hengstenberg, 1998). Consequently, EII could be immunologically targeted with antibodies, along with other known virulence factors to create a subunit vaccine.

Differential display PCR has successfully identified differentially expressed genes between prevalent and rare *S. aureus* strains, which may contribute to observed variations in virulence. Increased growth rate is one of many factors that contribute to *S. aureus* virulence in mastitis (Chesbro et al., 1968; White et al., 1980; Odierno et al., 1994). *Staphylococcus aureus* growth is in large part due to the availability of simple carbohydrates, such as glucose and lactose. Consequently, this study is the first to suggest that the lactose transporter, EII, may play a role in mastitis virulence as EII expression affects growth in a lactose-rich environment. Future research will focus on determining whether EII affects in vivo virulence. EII may be useful in the development novel therapeutic strategies against *S. aureus* mastitis.

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