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Identification of *Staphylococcus aureus* genes expressed during growth in milk: a useful model for selection of genes important in bovine mastitis?

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***Staphylococcus aureus* is a major cause of bovine mastitis. Since gene expression of many bacteria is known to be regulated by the environment, milk may play an important role in the regulation of the early steps in the pathogenesis of bovine mastitis by *S. aureus*. To get insight into the response of *S. aureus* to the milk environment, a Tn*917-lacZ* mutant library was generated and screened for genes specifically expressed during growth in milk. Twenty-eight mutants were identified and analysed. Four groups of genes were found, involved in cell-wall synthesis, nucleotide synthesis, transcriptional regulation and carbohydrate metabolism. A fifth group contained genes with hypothetical or unknown functions. Many of the genes identified belonged to biosynthetic pathways of *S. aureus* and other bacterial species which have also been shown to play a role *in vivo* as determined in murine infection models. Therefore, growth on milk may be an attractive model for the identification of genes preferentially expressed during bovine mastitis.**

Keywords: gene expression, mastitis, Tn*917-lacZ*, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is the most important causative agent of bovine mastitis, causing considerable economic losses in the dairy industry. The majority of the infections are subclinical, but the number of clinical cases is increasing. *S. aureus* infections are mainly treated with antibiotics, but such treatment is often unsuccessful. Therefore, new anti-staphylococcal therapies based on novel approaches are required. More knowledge about *S. aureus* gene expression during infection of the mammary gland will be helpful for that purpose.

It has been shown that bacteria, including *S. aureus*, respond to the complex *in vivo* environment by altering their gene expression (Finlay & Falkow, 1997; Lowe *et al.*, 1998). When *S. aureus* enters the mammary gland through the teat canal the bacteria are confronted with changing environmental conditions. Within a few hours of intramammary infusion of less than 100 *S. aureus* cells, large numbers of bacteria can be detected in the milk (Sutra & Poutrel, 1994). This suggests that *S. aureus* can easily adapt to, survive in and replicate in the bovine mammary gland. The ability of *S. aureus* to replicate rapidly in the mammary gland is considered to be important for establishing an infection. Because it is generally believed that replication of *S. aureus* takes place in close contact with milk, several investigators have studied growth of *S. aureus* in milk or milk whey *in vitro*. Densities of 10^8 c.f.u. ml^{-1} can be reached easily (Fang *et al.*, 1993). *S. aureus* cells grown in milk or milk whey *in vitro* become resistant to phagocytosis (Sutra *et al.*, 1990; Sandgren *et al.*, 1991) and show increased virulence in a mouse model (Mamo *et al.*, 1991), suggesting that a different set of genes becomes expressed during growth in milk. However, no infor-

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Abbreviations: β-gal, β-galactosidase; IVET, *in vivo* expression technology; STM, signature-tagged mutagenesis.

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mation is available on gene expression during growth in milk.

In order to identify genes that are specifically expressed during growth in milk, we generated a mutant library of *S. aureus* containing Tn*917-lacZ* insertions (Cheung *et al*., 1992). The transposon Tn*917-lacZ* contains a promoterless *lacZ* gene as a reporter gene, so that β-galactosidase (β-gal) expression is dependent on transcriptional fusions between *S. aureus* chromosomal promoters and the *lacZ* gene. The *S. aureus* transposon library was screened during growth on bovine milk and mutants were selected which expressed β-gal in milk but not in nutrient-rich growth media. In this way we have identified 28 *S. aureus* genes. Many of the genes identified belong to the same biosynthetic pathways as genes which were identified by *in vivo* approaches in various murine infection models (Mei *et al*., 1997; Coulter *et al*., 1998; Lowe *et al*., 1998).

METHODS

Bacterial strains, growth conditions and plasmid. *S. aureus* strain RN4220 was obtained from J. M. Patti (Centre for Extracellular Matrix Biology, Houston, TX, USA). *Escherichia coli* strains DH5α (Clontech) and XL2blue (Stratagene) were used for plasmid transformations and preparation of plasmid. *S. aureus* and *E. coli* strains were grown in Luria–Bertani (LB) medium (Sambrook *et al*., 1989) and on LB agar. When required, erythromycin (Erm, 10 μg ml^{-1}), tetracycline (Tet, 10 μg ml^{-1}) and ampicillin (50 μg ml^{-1}) were added to the media.

Two types of growth media were used for screening the *S. aureus* Tn*917-lacZ* transposon library. LB agarose plates contained LB with 1·5 % (w/v) agarose (Boehringer Mannheim), 0·5 % (w/v) glucose, 250 μg X-Gal ml^{-1} and 10 μg Erm ml^{-1}. Milk agarose plates contained fresh bovine milk, 1·5 % (w/v) agarose, 0·5 % (w/v) glucose, 0·5 % (w/v) casein enzymic hydrolysate (Casamino acids, Sigma), 250 μg X-Gal ml^{-1} and 10 μg Erm ml^{-1}. Prior to the preparation of the plates, the milk was incubated for 1 h at 80 °C. Nine volumes of the milk were mixed with one volume of a 15 % (w/v) agarose solution. To this solution glucose, Casamino acids, X-Gal and Erm were added. Glucose was added to the media to prevent endogenous β-gal expression by *S. aureus*. The Casamino acids were added to prevent selection of genes involved in biosynthesis of amino acids.

Plasmid pLTV1 (Camilli *et al*., 1990) was obtained from P. Youngman (University of Georgia).

Preparation of milk whey. Fresh bovine milk was centrifuged for 30 min at 13000 ***g***. After removal of the fat layer, 40 μg ml^{-1} rennin (Rademaker BV, The Netherlands) was added and the solution was incubated for 2 h at 37 °C and centrifuged twice for 30 min at 13000 ***g***. The supernatant was subsequently sterilized through a 0·45 μm Millipore filter and stored at −20 °C.

Generation of the *S. aureus* Tn*917-lacZ* library. Plasmid pLTV1 was transformed to *S. aureus* strain RN4220 by electroporation (Schenk & Laddaga, 1992). Transformed cells were selected at 30 °C on LB agar plates containing Tet (10 μg ml^{-1}). Subsequently, the colonies were suspended in 10 ml LB with 2 μg Erm ml^{-1} to a density of 10^{10} bacteria ml^{-1} and incubated for 6 h at 43 °C in a shaking waterbath. Then, the suspension was transferred to 100 ml LB containing 5 μg Erm ml^{-1} and incubated overnight at 43 °C in a shaking waterbath. One hundred millilitres of the overnight culture was diluted fivefold in LB containing 5 μg Erm ml^{-1} and incubated for 6 h at 43 °C in a shaking waterbath. From this culture 10 ml was diluted 80-fold in LB containing 5 μg Erm ml^{-1} and incubated overnight in a shaking waterbath of 43 °C. Aliquots of this culture were stored at −70 °C.

Screening of the *S. aureus* library. The *S. aureus* library was plated on milk agar plates and individual colonies were plated on LB or milk agarose plates. Colonies that were white on LB and blue (pale to dark blue) on plates containing milk were selected. All selected mutants were rescreened and characterized further.

Cloning and sequencing of *S. aureus* DNA sequences upstream of Tn*917-lacZ* insertions. Cloning of DNA located upstream of the transposon insertion was essentially done as described by Camilli *et al*. (1990). Chromosomal DNA of *S. aureus* RN4220 mutants was cut with *Eco*RI, ligated and used to transform *E. coli* DH5α or XL2blue cells. Plasmid DNA for sequencing was isolated using the Wizard plus SV miniprep DNA purification system (Promega). DNA sequences were determined by a 373A DNA Sequencing System (Applied Biosystems). Samples were prepared by use of an ABI/PRISM dye termination cycle sequencing ready reaction kit (Perkin-Elmer). Sequence data were assembled and analysed using the BLASTX program (Altschul *et al*., 1990). The primer used in the sequencing reactions was 5′-CACAATAGAGAGATGTCACCG-3′ and corresponded to position 84–104 in the Tn*917* sequence.

Southern blotting and hybridization. Chromosomal DNA of *S. aureus* was isolated essentially as described by Sambrook *et al*. (1989). To lyse the *S. aureus* cells, lysozyme was replaced by lysostaphin (10 μg ml^{-1}; Sigma). Southern blot analysis of transposon insertions in the *S. aureus* chromosome was performed using the pLTV1 plasmid as a probe. Labelling of the probe and hybridization of the blots was essentially done as previously described (Smith *et al*., 1995).

Phage transduction. The transposons of the selected mutants were transferred to the *S. aureus* strain 8325-4 background using the transducing phage φ*85* as described by Novick (1991). The transductants were selected for Erm resistance. To discriminate the original RN4220 mutant from the transduced 8325-4 mutant, the haemolytic phenotype was determined on agar plates containing 5 % washed bovine red blood cells. Three individual transductants of each mutant were tested for expression of the *lacZ* gene on LB and milk plates.

Growth analysis of transduced mutants. The growth rate of the selected mutants was determined by measuring the increase in the OD_{600}. Overnight cultures in LB or milk whey were diluted 1/50 in LB or milk whey in microtitre plates. The microtitre plates were incubated at 37 °C and the OD_{600} was measured as a function of time in a microplate reader. The growth rate of mutants that were impaired in their ability to grow was redetermined using 100 ml cultures in Erlenmeyer flasks.

β-Gal expression in liquid milk whey. β-Gal assays were performed using the chemiluminescent β-Gal Reporter Gene Assay (Boehringer Mannheim). Briefly, 1 ml aliquots of *S. aureus* cells grown in milk whey or LB were sampled as a function of time, centrifuged for 10 min at 3000 ***g*** and washed once in ice cold PBS (0·01 M, pH 7·2). The pellets were frozen at −20 °C for later analysis. The frozen cell samples were thawed and lysed in 200 μl lysis buffer containing 20 μg

lysostaphin ml^{-1} for 30 min at 37 °C. Samples of 50 µl were tested in the chemiluminescent assay in triplicate. The reaction time was carried out for 60 min at room temperature in white 96-well microtitre plates (Greiner). Chemiluminescent signals were detected in a Victor 1420 multilabelcounter (Wallac Oy). β-Gal activity was expressed as counts per second (c.p.s.) per OD_{600} unit.

RESULTS AND DISCUSSION

Construction of the transposon library

The objective of our study was the screening of a mutant *S. aureus* library for expression of *lacZ* under the control of *S. aureus* promoters. To construct such a library, we selected an *S. aureus* strain which is able to persist and replicate in the udder and which is able to express *lacZ* in a way that results in blue colonies on X-Gal plates. *S. aureus* strain RN4220 induced an inflammatory response and the bacteria persisted and replicated in the mammary gland for at least several days (data not shown). Therefore, strain RN4220 was used for the construction of the Tn*917-lacZ* library. To determine the transposition frequency the library was plated on plates containing Erm or Tet. Over 99 % of the cells were Erm resistant and Tet sensitive, indicating the insertion of the transposon into the chromosome and the loss of pLTV1. Southern blotting and hybridization experiments showed that 14 out 18 mutants analysed had insertions at unique positions (results not shown). Four mutants showed identical hybridization patterns, indicating the presence of at least one hot spot. As well as hot spots, hot-spot regions have also been found by Tn*917* insertions in Gram-positive bacteria (Youngman, 1987; Camilli *et al*., 1990). The presence of these hot-spot regions in *S. aureus* cannot be excluded, but broad coverage of the genome by Tn*917* insertions has been reported (Coulter *et al*., 1998). Based on our data, the Tn*917* library seemed to contain enough variation to allow the identification of various differentially expressed *S. aureus* genes during growth in milk.

Identification of milk-induced genes

An appropriate dilution of the transposon library was plated on milk agarose plates containing X-Gal. About 150 blue colonies were selected and plated on LB and milk agarose plates. Transposon mutants showing increased levels of β-gal expression on plates containing milk were selected and rescreened on the two different growth media. Finally, 28 mutants were selected and characterized further. The colour of the selected colonies on milk plates containing X-Gal varied from pale blue to dark blue, indicating that the different mutants had various levels of β-gal expression. β-Gal activities on milk plates and milk whey plates were comparable (results not shown).

S. aureus strain RN4220 is chemically mutagenized. To exclude the possibility that secondary mutations elsewhere on the genome of this strain had an effect on β-gal expression, bacteriophage φ85 was used to transduce the Tn*917-lacZ* insertions into *S. aureus* strain 8325-4. The transductants were subsequently tested for their β-gal activity (Table 1). No major differences were observed between the β-gal activity of the 8325-4 transductants (designated SMI01T to SMI43T) and the original RN4220 mutants (designated SMI01 to SMI43; results not shown). This strongly indicates that the genes selected in strain RN4220 are induced by environmental conditions and are not the result of secondary mutations in the genome of this strain.

In the primary selection, glucose was added to prevent expression of endogenous *S. aureus* genes encoding β-gal (Oskouian & Stewart, 1990). Moreover, Casamino acids were added to prevent the selection of genes involved in amino acid biosynthesis. However, since both glucose and Casamino acids may affect the bacterial gene expression (Ohlsen *et al*., 1997; Chan & Foster, 1998), we subsequently determined the β-gal activity of the selected mutants on LB and milk plates without glucose and Casamino acids. No effects of glucose and Casamino acids on β-gal expression were observed (results not shown). This indicates that the expression of the genes selected in this study was not regulated by glucose or Casamino acids.

β-Gal activities in liquid milk whey and LB

We next tested whether the differences in β-gal expression observed on plates could also be measured after growth in liquid media. For this purpose, we measured β-gal activities in liquid milk whey and LB. The majority of the mutants (23/28) showed enhanced β-gal activities after growth in milk whey compared to growth in LB (Table 1). In five mutants the differences between β-gal expression were more pronounced after growth on plates than after growth in liquid media. This may be due to the type of growth medium (solid vs liquid) or the growth phases in which the β-gal activity was determined (colonies vs exponential-phase cultures).

Growth rate of the selected mutants in milk whey

To investigate whether the transposon insertions affected the ability to grow in milk whey, overnight cultures of the mutant strains in LB or milk whey were diluted in LB medium and milk whey and incubated in microtitre plates at 37 °C. The OD_{600} was measured as a function of time. All 28 mutants showed the same growth rate in LB medium. However, three mutants showed a reduced growth rate in milk whey. This was confirmed by culturing in Erlenmeyer flasks (Fig. 1a). Sequence analysis (see below) showed that these mutants were impaired in their purine biosynthetic pathway. The addition of 1·0 mM adenine to milk whey partly restored the growth rate of the mutants SMI05T (identical to SMI32T) and SMI06T (Fig. 1a) and reduced the expression of β-gal (15- and 25-fold, respectively; Fig. 1b). Similar results were obtained by Klarsfeld *et al*. (1994) for *Listeria monocytogenes*, in which insertional inactivation of *pur* genes also inhibited growth in a defined

Table 1. Milk-expressed *S. aureus* genes

Gene class	Mutant*	Similar to†	Percentage identity (positives)	β-Gal activity on:		Ratio of β-gal activity‡	Presumed function, SWISS-PROT TREMBL numbers and PROSITE motifs
				LB plates	Milk plates		
I. Peptidoglycan/lysine biosynthesis	SMI01T, SMI08T	*lysA* (*Bsu*)	41 (59)	−	+	2	Lysine biosynthesis/peptidoglycan biosynthesis
	SMI09T	*asd* (*Bsu*)	50 (69)	−	+	7	Lysine biosynthesis/peptidoglycan biosynthesis
	SMI26T	*asd* (*Bsu*)	53 (71)	−	+	12	Lysine biosynthesis/peptidoglycan biosynthesis
	SMI13T	*lysC* (*Mtu*)	43 (59)	−	++	54	Lysine biosynthesis/peptidoglycan biosynthesis
	SMI29T	*dapA* (*Mja*)	35 (51)	−	+	9	Lysine biosynthesis/peptidoglycan biosynthesis
	SMI12T	*femB* (*Sau*)	98 (98)	+	++	1	Peptidoglycan cross-linking
	SMI18T	*murZ* (*Bsu*)	43 (59)	+/−	+	2	Peptidoglycan biosynthesis
II. DNA biosynthesis	SMI05T, SMI32T	*purH* (*Bce*)	60 (80)	−	++	1250	Purine biosynthesis
	SMI06T	*yexA* (*Bsu*)	33 (64)	−	++	1117	Purine biosynthesis, P12049
	SMI20T	*purD* (*Hpy*)	38 (59)	−	++	196	Purine biosynthesis
	SMI07T	*pyrP* (*Bca*)	56 (66)	−	++	18	Uracil permease
III. Transcriptional regulators	SMI02T	*phoR* (*Bsu*)	51 (72)	−	++	1	Histidine phosphokinase, regulation gene expression under phosphate-limiting conditions
	SMI33T	*comK* (*Bsu*)	43 (62)	−	++	12	Transcriptional regulation of genetic competence
IV. Carbohydrate metabolism	SMI15T	*F45e1.3* (*Cel*)	32 (54)	−	+	3	Carbon metabolism, U28732
V. Hypothetical genes	SMI44T, SMI46T	Staphopain gene (*Sau*)	47 (66)	−	++	1680	Hypothetical thiol-induced protease P81297, PS00639
	SMI019T	*ykd* (*Bsu*)	46(72)	+	+	1·6	Unknown, hypothetical 23·6 kDa protein, P94357
	SMI04T	None		−	+	5	Unknown
	SMI016T	None		−	+	1	Unknown
	SMI017T	None		−	+	2	Unknown
	SMI035T	None		−	+	16	Unknown
	SMI040T	None		−	+	10	Unknown
	SMI024T	None		+/−	+	1	Unknown
	SMI025T	None		+	++	1	Unknown
	SMI027T	None		−	++	14	Unknown
	SMI043T	None		−	++	90	Unknown

* Sequence analysis was done with cloned DNA from the initial RN4220 mutants. β-Gal activity was determined with transductants of strain 8325-4.

† *Bsu*, *Bacillus subtilis*; *Mtu*, *Mycobacterium tuberculosis*; *Mja*, *Methanococcus jannaschii*; *Sep*, *Staphylococcus epidermidis*; *Bce*, *Bacillus cereus*; *Hpy*, *Helicobacter pylori*; *Bca*, *Bacillus caldolyticus*; *Ech*, *Erwinia chrysanthemi*; *Cel*, *Caenorhabditis elegans*; *Sau*, *Staphylococcus aureus*.

‡ Induction is expressed as the ratio of β-gal activity of bacteria cultured to OD_{600} 0·5 in milk whey to that of bacteria cultured to OD_{600} 0·5 in LB medium. The cells of strains SMI05T, SMI06T and SMI032T were harvested at an OD_{600} of approximately 0·15.

medium. However, in that study the addition of adenine had a more pronounced effect on the growth rate and the β-gal activity. Mutant SMI20T, mutagenized in the *purD* gene, showed no reduced growth rate in milk whey, but the addition of adenine reduced the β-gal activity eightfold (Fig. 1b). As a control the effect of adenine on the β-gal activity of mutant SMI07T was determined (Fig. 1b). This mutant, mutagenized in the *pyrP* gene encoding a pyrimidine transporter, showed no decreased β-gal expression after addition of adenine.

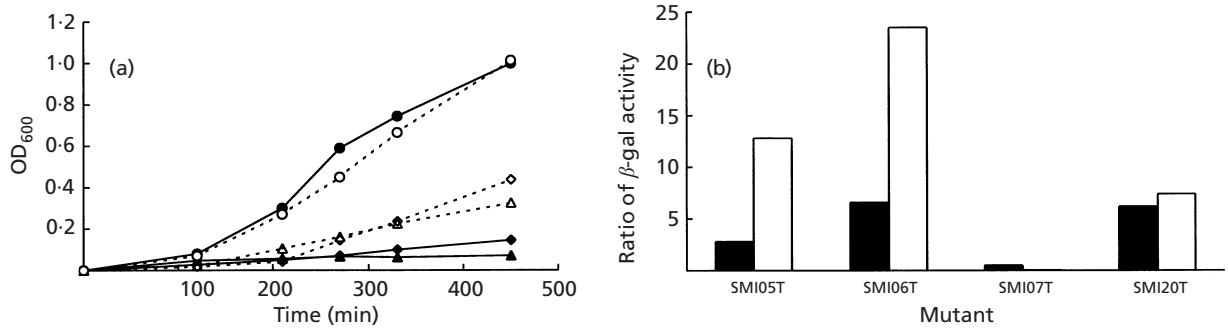

Fig. 1. Effect of adenine on the growth rate (a) and β-gal activity (b) of *S. aureus* strains. (a) Growth rates of strains SMI05T (triangles), SMI06T (diamonds) and 8325-4 (circles) in milk whey without adenine (solid lines and closed symbols) or in milk whey with 1 mM adenine (dashed lines and open symbols). (b) β-Gal activity of bacteria cultured for 210 (solid bars) and 330 (open bars) min at 37 °C in milk whey without adenine expressed as a ratio relative to the β-gal activity with adenine.

Cloning and sequence analysis of flanking chromosomal DNA regions

Southern blotting experiments of *Eco*RI-digested chromosomal DNAs of the selected mutants, probed with the pLTV1 vector, showed that all mutant strains contained a single transposon insertion (data not shown). Chromosomal DNA regions flanking the *lacZ*-proximal end of the transposon were cloned in *E. coli*. The plasmids were isolated and the upstream flanking DNA regions were sequenced. For each clone approximately 300 bp of sequence was obtained. The sequences were analysed for homology to genes present in data libraries, and were classified based on the (hypothetical) functions of the genes (Table 1).

I. The first class, of eight mutants, comprised insertions in genes with homology to genes involved in lysine and/or peptidoglycan synthesis. Lysine is present in proteins as well as in the tetrapeptide cross-bridges of the cell wall peptidoglycan of *S. aureus* (Seligman & Pincus, 1987). *lysA* encodes diaminopimelate decarboxylase and is involved in the conversion of diaminopimelate to lysine (Wehrmann *et al*., 1998). *S. aureus asd* and *lysA* genes were also identified after using STM in murine infection models (Mei *et al*., 1997; Coulter *et al*., 1998). This means that mutations in genes of this pathway cause attenuation *in vivo*. Mutant SMI12T contained a transposon insertion in the *femB* gene of *S. aureus* (Berger-Bachi *et al*., 1992). The *femB* gene is involved in formation of the peptidoglycan pentaglycine cross-bridges (Ehlert *et al*., 1997). *femB* was also identified by STM, and *femB* knockout mutants were strongly attenuated in murine infection models (Mei *et al*., 1997; Coulter *et al*., 1998). Mutant SMI18 contained a transposon insertion in a gene that shares homology to *murZ* of *Bacillus subtilis*, which is also involved in peptidoglycan synthesis (Marquardt *et al*., 1992). After using IVET an *S. aureus* gene homologous to *murC* of *B. subtilis* was identified; this gene was induced in a murine infection model (Lowe *et al*., 1998). The *mur*C gene is also involved in peptidoglycan biosynthesis. Both IVET and STM studies showed that *S. aureus* genes involved in peptidoglycan synthesis play a role *in vivo* as determined in murine infection models. By selecting for *S. aureus* genes specifically expressed on milk, genes that may be involved in peptidoglycan biosynthesis were selected with a relatively high frequency (6/25). This suggests that enhanced expression of genes involved in peptidoglycan biosynthesis is required for growth or persistence of *S. aureus* in milk. Moreover, after growth of *S. aureus* in milk whey, the bacteria were very difficult to lyse by using lysostaphin (data not shown). Because lysostaphin acts on the peptidoglycan structure, this observation suggests that the peptidoglycan layer was thicker or the composition was changed. It has been reported that milk-whey-grown *S. aureus* strains were enhanced in virulence (Mamo *et al*., 1991). However, it has not been investigated whether this enhanced virulence was due to changes in the peptidoglycan layer.

II. The genes of the second group of mutants, SMI05, SMI06, SMI07, SMI20 and SMI32, showed identity to genes involved in nucleic acid biosynthesis. SMI05 and SMI32 showed identity to the *purH* gene of *Bacillus cereus* and SMI20 to the *purD* gene of *Helicobacter pylori*. *L. monocytogenes purD* and *purH* genes were also found to be induced in eukaryotic cells (Klarsfeld *et al*., 1994), and *S. aureus* and *Streptococcus pneumoniae* genes sharing homology with genes in the same biosynthetic pathway were also identified by STM (Mei *et al*., 1997; Coulter *et al*., 1998; Polissi *et al*., 1998). *pur* mutants of *Salmonella* and *Pseudomonas* were attenuated *in vivo* (Mahan *et al*., 1993; Wang *et al*., 1996). The gene of mutant SMI06 is homologous to the *yexA* gene of *B. subtilis*. *yexA* is a hypothetical gene of the *pur* operon of this organism. As discussed above, the mutants SMI05T and SMI06T had a decreased growth rate in milk whey. This suggests that *pur* genes play an important role during replication in milk. The sequences of mutant SMI07 were homologous to *pyrP* of *Bacillus caldolyticus*. In this organism the *pyrP* gene encodes a membrane transporter of pyrimidines (Ghim & Neuhard, 1994).

III. The third class of mutants had insertions in genes homologous to genes with a transcriptional regulatory function. The gene impaired in strain SMI02 had strong similarity at the protein level to *phoR* of various bacterial species. *phoR* encodes the sensor protein which is part of a two-component signal transduction system (Seki *et al*., 1988). Two-component regulatory systems play important roles in the adaptive responses of many bacteria to environmental changes (Finlay & Falkow, 1997). The *phoR/phoP* operon is upregulated under phosphate-limiting conditions. Under these conditions the production of alkaline phosphatases and phosphate-transport proteins is increased by the transcriptional activator *phoP*. In *E. coli* at least 30 genes are regulated by this system (Wanner, 1993). An *E. coli* mutant containing a transposon insertion in the *pst/phoU* region is hyperinvasive for epithelial cells (Sinai & Bavoil, 1993), suggesting that genes of the *pho* regulon may be involved in pathogenesis. In *Bacillus subtilis*, *phoR/phoP* is part of a complex network of signal-transduction systems (Birkey *et al*., 1998). In addition, the *phoH* gene of *S. pneumoniae*, also part of the *pho* regulon, was identified by STM (Polissi *et al*., 1998). Therefore, the insertional mutation in strain SMI02 may have an important influence on the ability of *S. aureus* to adapt to the udder environment. Mutant SMI33 had an insertion in a gene homologous to *comK* of *B. subtilis*, which encodes a transcriptional regulator involved in genetic competence. The signals involved in competence development all converge at the level of *comK* expression (van Sinderen *et al*., 1995). Therefore, *comK* has a central role in the transition from exponential phase to the genetic competence phase of *B. subtilis*. Further characterization is needed to elucidate if this *S. aureus* gene also has a central regulatory function.

IV. The gene identified in mutant SMI15 showed a strong homology to a hypothetical gene of the nematode *Caenorhabditis elegans* and to phosphoglycolate phosphatases of various bacterial species. The product of the *C. elegans* gene also has homology to phosphoglycolate phosphatases of various organisms. Phosphoglycolate phosphatase is a key enzyme of glycolate metabolism in autotrophic organisms (Schaferjohann *et al*., 1993).

V. Two mutants, SMI44 and SMI46, had insertions in a gene which showed homology to an *S. aureus* gene encoding a protein called staphopain. Staphopain contains a domain with homology to thiol-activated proteases. This domain was also present in the identified sequences of strains SMI44 and SMI46, suggesting that this gene also encodes a thiol-activated protease. Thiol-activated proteases, or cysteine proteases, are present in both eukaryotic and prokaryotic organisms and some are known to play an important role in virulence. For instance, the *speB* gene of *Streptococcus pyogenes* encodes a cysteine protease and is a major virulence factor (Podbielski *et al*., 1999). Recently, a cysteine proteinase was purified from an *S. aureus* strain derived from a diseased chicken (Takeuchi *et al*., 1999). The N-terminal amino acid sequence of this protease was also strongly homologous to the staphopain sequence.

Nine milk-expressed genes had no sequence homology with any sequence in the databases. These mutants had different levels of β-gal expression on milk agarose plates.

Concluding remarks

Growth on milk, which partly mimics the early steps in the pathogenesis of *S. aureus* mastitis, was used for the identification of specifically expressed *S. aureus* genes. Several genes that may contribute to persistence and replication in the bovine mammary gland were identified. The significance of this *in vitro* strategy is further demonstrated by the observation that some of the selected genes were also found after using IVET or STM. Therefore, this *in vitro* approach is likely to be a fast and simple method for the selection of genes important in the pathogenesis of *S. aureus* mastitis. In addition, this model may be useful for identification of novel growth-inhibitory compounds for use as therapeutic agents for bovine mastitis.

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