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Dual Role of the Oligopeptide Permease Opp3 during Growth of *Staphylococcus aureus* in Milk▽

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***Staphylococcus aureus* RN6390 presents a diauxic growth in milk, due to amino acid limitation. Inactivation of the oligopeptide permease Opp3 (dedicated to the nitrogen nutrition of the strain) not only affects the growth of the strain but also results in reduced expression levels of three major extracellular proteases.**

Staphylococcus aureus is a ubiquitous gram-positive pathogen commonly found in the environment. Some *S. aureus* strains are able to produce enterotoxins that cause food poisoning (6). In France, contaminated dairy products are the main source of staphylococcal food-borne disease outbreaks (3). Thus, the development of this bacterium in milk is a major concern for the safety of dairy products. Our objective was to evaluate the growth of *S. aureus* in milk, with special attention focused on the role of the peptide transport systems.

Opp3 is involved in amino acid supply during growth of *S. aureus* RN6390 in milk. *S. aureus* strain RN6390 (7) was grown at 37°C in reconstituted skim milk (Nilac; Duiven, The Netherlands) with shaking (200 rpm). The time courses of cell populations for seven independent replicate cultures were evaluated by plating culture dilutions after disrupting bacterial clumps with an Ultra Turrax (IKA, Staufen, Germany).

Linear regression analysis performed for each growth experiment discarded the hypothesis of a single exponential growth phase, with the standard errors of the estimate in the same range as the growth rates (from 0.70 to 1.19 h^{-1}). *S. aureus* presented a diauxic growth in milk, with two distinct exponential growth phases separated by a short transition period (Fig. 1). The mean growth rates of the two exponential phases were 1.35 ± 0.03 h^{-1} and 0.60 ± 0.04 h^{-1} (means for seven determinations ± confidence limits at P values of 0.95), with standard errors of the estimate lower than 0.23 and 0.17 h^{-1}, respectively.

When milk was supplemented with a mixture of 18 amino acids (10), growth of *S. aureus* RN6390 was clearly modified (Fig. 1). Only one single exponential growth phase was observed ($\mu = 1.70$ h^{-1}), whereas the final population was not affected (ca. 5×10^9 CFU · ml^{-1}). These results suggest that the amino acid composition, rather than the concentration of amino acids present in nonsupplemented milk, accounts for the growth limitation of the strain.

Five putative peptide transport systems are present in *S. aureus*: four ABC transporters, named Opp (oligopeptide permease), and one permease, DtpT. Among those, only Opp3 and DtpT exert a nutritional function, ensuring the import of tri- to octapeptides and di- and tripeptides, respectively (5). The role of these two peptide transport systems in growth in milk was evaluated by measuring the growth of *opp3* and *dtpT* mutant strains. All deletion mutants were constructed by double crossover using the pMad vector as previously described (1, 5). While the *dtpT* mutant strain grew similarly to the wild-type strain, the growth of the *opp3* mutant strain was altered, as the duration of the transition period was fivefold longer (Fig. 2). This suggests that oligopeptides are used as a source of amino acids at this stage of growth. The double *opp3 dtpT* mutant presented only the first exponential phase and a strongly reduced final population (about 10-fold), indicating that di- and tripeptides are a significant source of amino acids when the strain is unable to import oligopeptides. These results were further supported by growths in milk supplemented with a mixture of 18 amino acids in which all mutant strains grew as the wild-type strain (data not shown).

The growth in milk of the *opp1 opp2 opp4 dtpT* mutant strain was comparable to that for the wild type (data not shown), confirming the absence of significant nutritional function for Opp1, Opp2, and Opp4 systems, as reported previously (4).

Altogether, these results allow us to postulate the following three-stage scheme of nitrogen nutrition in *S. aureus*: (i) first, the bacteria utilize free amino acids from milk to reach a cell density of about 3×10^8 to 5×10^8 CFU ml^{-1}; (ii) the pool of free amino acids in milk becomes too low, resulting in a transient growth arrest (this is supported by the fact that none of the amino acids required by *S. aureus* for an efficient growth, namely, Glu, Cys, Leu, Met, Gly, Val, Thr, Arg, and Lys [3], could be detected anymore by high-performance liquid chromatography in their free form in the milk cultures at this stage of growth [data not shown]); and (iii) a complementary source of amino acids supplied as peptides allows bacteria to restart growth and to reach high cell densities (about 5×10^9 CFU · ml^{-1}).

Opp3 participates in extracellular protease regulation during growth in milk. Milk coagulation was observed during the transition period of the wild-type strain. As the culture was

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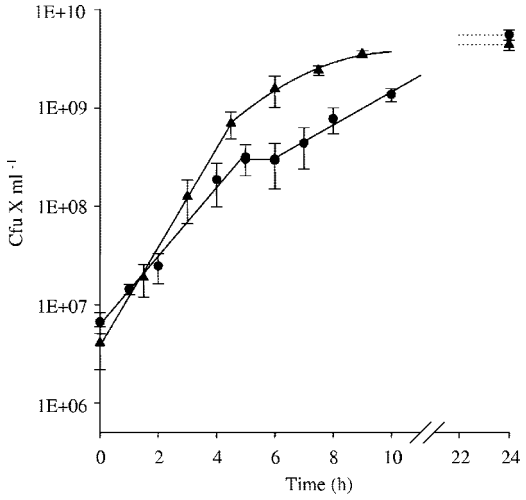

FIG. 1. Growth of the *S. aureus* RN6390 wild-type strain in milk (●) or in milk supplemented with a mixture of 18 amino acids (▲).

stirred, *S. aureus* developed a respiratory metabolism that did not result in a decrease of pH. Consequently, milk clotting was most probably due to casein proteolysis. This phenomenon was delayed in the cultures of *opp3* and *opp3 dtpT* mutant strains (1.5 h and 6 h, respectively). As the cell densities of the strains were in similar ranges at this growth stage (Fig. 2), this delay might be caused by reductions in the proteolytic activities of the mutant strains. To evaluate this hypothesis, the extracellular proteolytic activities of the wild-type and *opp3* mutant strains were evaluated. Supernatants from transition period cultures were settled onto cellulose disks placed onto agar (1.5%) plates containing 3% casein. After incubation at 37°C, the halo of proteolysis (white precipitate of casein) observed with the *opp3* mutant was smaller than that of the wild-type strain, indicating a lower proteolytic activity (data not shown). Ten extracellular proteases (SspA and B; Aur; ScpA; and SplA, B, C, D, E, and F) have been identified in *S. aureus*. To test a putative deregulation in the transcription of one or

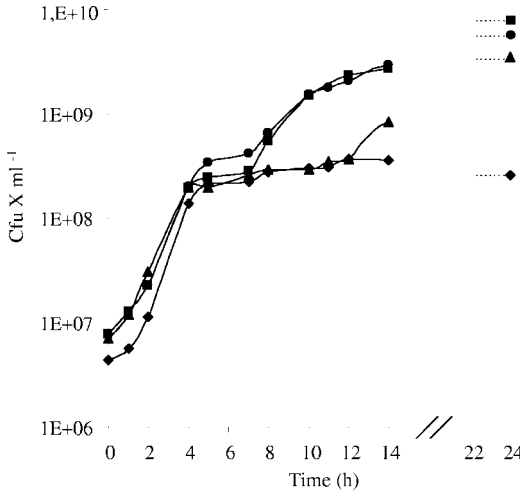

FIG. 2. Growth of *S. aureus* RN6390 wild-type (●) and *dtpT* (■), *opp3* (▲), and *opp3 dtpT* (◆) mutant strains during growth in milk.

TABLE 1. Oligonucleotide primers used for quantitative PCR

Gene	Primer (5′–3′)	
	First	Second
gyrB	ATCGGTGGCGACTTTGATCT	CGGCATCAGTCATAATGACGATT
aur	TGAAGATGTCTACACACCTG GAAAA	AATTGTTCTGGGTTTGACATGCT
sspA	ACTTGTGAGTTCTCCAGCA GCAA	TGACTGCGTTTGTTGTGGAT
sspB	TGTAGGAATTATGATCCTTG CACAA	CAACTGCTAGCGCATGTCCTAA

several of the proteases encoded by genes in the *opp3* mutant strain, quantitative real-time PCR on the *sspA*, *aur*, *scpA*, and *splA* genes was performed (*sspB* and the *splB* to *-F* genes are cotranscribed with *sspA* and *splA*, respectively). RNAs and cDNA were prepared as previously described (5), and quantitative PCR was performed by using a SYBR green master mix (Applied Biosystems) with 2 ng of cDNA (primers are listed in Table 1). *gyrB* was validated as a stably expressed gene under our conditions and used to normalize the quantity of each cDNA tested. Inactivation of *opp3* did not affect the expression levels of *scpA* and *splA* (data not shown). However, the levels of *sspA* and, to a lower extent, *aur* transcripts were reduced in the *opp3* mutant (Fig. 3). As expected from the genetic organization of *ssp* genes, the *sspB* transcript level was also decreased (data not shown). We further observed a 1.5-h delay in milk coagulation in the culture of an *sspA* isogenic mutant strain (8), compared to the level for the parental RN6390 strain, confirming the implication of Ssp protease activity in the degradation of milk proteins (data not shown). Altogether, these results indicate that the delay in coagulation with the *opp3* mutant strain results from a reduction in proteolytic activity caused at least by a decrease in *ssp* transcription. It is worth noting that no delay in coagulation could be observed between wild-type and *opp3* mutant strains when milk was supplemented with a mixture of amino acids. This suggests that Opp3 could be involved in environmental sensing by modulating intracellular amino acid pools acting on pleiotropic regu-

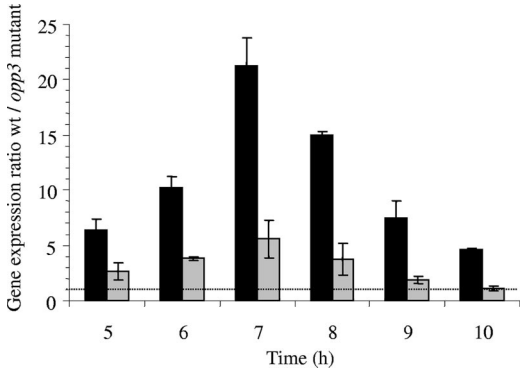

FIG. 3. Effect of *opp3* deletion on expression of *sspA* (black bars) and *aur* (gray bars) by *S. aureus* RN6390 grown in milk. Expression levels of *sspA* and *aur* in the *S. aureus* wild-type and *opp3* mutant strains were estimated by quantitative PCR. For each strain and each time point, gene expression was normalized using *gyrB* as the stably expressed reference gene. Results are presented as ratios of gene expression (wild-type [wt] strain/*opp3* mutant). Vertical bars indicate the standard deviations.

lators, as already demonstrated for other bacterial Opp systems (2, 4, 9). The amino acids and the regulator involved in this mechanism remain to be elucidated.

In conclusion, peptides transported by Opp3 play a dual role during growth of *S. aureus* in milk, supplying bacteria with nitrogenous nutrients and influencing expression of genes encoding three major extracellular proteases, most probably via an environmental sensing mechanism. Thus, Opp3 might represent a new target for controlling *S. aureus* development in dairy products.

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