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# Only One of Four Oligopeptide Transport Systems Mediates Nitrogen Nutrition in *Staphylococcus aureus*▽

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**Oligopeptides internalized by oligopeptide permease (Opp) transporters play key roles in bacterial nutrition, signaling, and virulence. To date, two *opp* operons, *opp-1* and *opp-2*, have been identified in *Staphylococcus aureus*. Systematic in silico analysis of 11 different *S. aureus* genomes revealed the existence of two new *opp* operons, *opp-3* and *opp-4*, plus an *opp-5A* gene encoding a putative peptide-binding protein. With the exception of *opp-4*, the *opp* operons were present in all *S. aureus* strains. Within a single strain, the different *opp* operons displayed little sequence similarity and distinct genetic organization. Transcriptional studies showed that *opp-1*, *opp-2*, *opp-3*, and *opp-4* operons were polycistronic and that *opp-5A* is monocistronic. We designed a minimal chemically defined medium for *S. aureus* RN6390 and showed that all *opp* genes were expressed but at different levels. Where tested, OppA protein production paralleled transcriptional profiles. *opp-3*, which encodes proteins most similar to known peptide transport proteins, displayed the highest expression level and was the only transporter to be regulated by specific amino acids, tyrosine and phenylalanine. Defined deletion mutants in one or several peptide permeases were constructed and tested for their capacity to grow in peptide-containing medium. Among the four putative Opp systems, Opp-3 was the only system able to provide oligopeptides for growth, ranging in length from 3 to 8 amino acids. Dipeptides were imported exclusively by DtpT, a proton-driven di- and tripeptide permease. These data provide a first complete inventory of the peptide transport systems *opp* and *dtpT* of *S. aureus*. Among them, the newly identified Opp-3 appears to be the main Opp system supplying the cell with peptides as nutritional sources.**

Oligopeptide permeases (Opp) have been identified in numerous gram-negative and -positive bacteria. These transport systems belong to the superfamily of highly conserved ATP-binding cassette transporters (44). Typically, Opp importers comprise a complex of five proteins. The oligopeptide-binding protein OppA is responsible for the capture of peptides from the external medium. OppA is a periplasmic protein in gram-negative bacteria, whereas it is a lipoprotein in gram-positive bacteria. Two integral transmembrane proteins, OppB and OppC, form a channel through the membrane used for peptide translocation. Two membrane-bound cytoplasmic ATP-binding proteins, OppD and OppF, provide energy for peptide transport.

At a genetic level, the five *opp* genes encoding the transporter are usually organized in an operon, *oppABCDF*. However, gene organization can vary, or one of the *opp* genes may be absent (19). Moreover, the number of peptide-binding protein-encoding genes associated with the *opp* operon is variable from one system to another. The *opp* operons of *Listeria monocytogenes* or *Lactococcus lactis* contain a single copy of *oppA* (6, 46), whereas two, three, and five distinct *oppA* genes are associated with the *opp* operons of *Escherichia coli* (34), *Streptococcus thermophilus* (16), and *Borrelia burgdorferi* (24), respectively.

Opp systems are generally regulated at the transcriptional level. Particular intracellular pools of amino acids, e.g., leucine and alanine in *E. coli* (2) or branched-chain amino acids in *L. lactis* (20), regulate *oppA* expression. Environmental changes have also been shown to influence *opp* expression. For example, transcription of *oppA* genes is induced by a temperature down-shift in *L. monocytogenes* (6) and *Bacillus subtilis* (7); expression of the *E. coli opp* operon is up-regulated under anaerobic conditions (2).

A variety of roles have been described for the bacterial Opp systems. The most obvious one is to supply bacteria with essential amino acids, as demonstrated for lactic acid bacteria (15, 25). Duplication of OppA and/or Opp homologues could be explained as a means of increasing peptide transport efficiency and thereby optimizing nutritional function. Besides nutrition, Opp systems might also be involved in various functions such as cell wall turnover in *E. coli* (34) or peptide-mediated signaling in *B. subtilis* and *Enterococcus faecalis* (28). Opp systems also play a role in virulence of some gram-positive pathogenic bacteria by transporting a specific peptide (called a pheromone) that activates a pleiotropic virulence regulon, as demonstrated in the case of *Bacillus thuringiensis* (18, 43), or by stimulating adherence of pathogenic streptococci to human cells (10, 40).

In *Staphylococcus aureus*, two putative oligopeptide transport systems, Opp-1 and Opp-2 were suggested to play a role in different infection models (5, 9, 29). *S. aureus* is a remarkably versatile pathogen, responsible for a broad spectrum of

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TABLE 1. Primers used for deletion of *opp*-like operons and the *dtpT* gene of *S. aureus* RN6390

| Operon or gene to delete | Primer name | Sequence (5′→3′)[a] |
|---|---|---|
| *opp-1* | BoxA-*opp1*-5′ | CGGGATCCCCTGAAGGACCGATAACGATGACAC |
| | BoxA-*opp1*-3′ | CGCAACACGTTGCGCCTCTCCGCCCTGATGCAAGTAACATTGCACTC |
| | BoxB-*opp1*-5′ | GTTACTTGCATCAGGGCGGAGAGGCGCAACGTGTTGCGATTGCGCGTGC |
| | BoxB-*opp1*-3′ | CGGGATCCCCCGCATTCGCGAATCCTAAACTC |
| *opp-2* | BoxA-*opp2*-5′ | CGGGATCCGTCTCCTAAACGATAATGTTACGA |
| | BoxA-*opp2*-3′ | GCCATCCTTAAGAACGCCCCCTCCTTGTGTAAGTTACTAACAC |
| | BoxB-*opp2*-5′ | CTTACACAAGGAGGGGGCGTTCTTAAGGATGGCATGATAGTCG |
| | BoxB-*opp2*-3′ | CGGGGATCCGGACCATCAGAAGCATACACACACGC |
| *opp-3* | BoxA-*opp3*-5′ | CGGGATCCGGTCACTTACTTGGTGCAACAGGTGG |
| | BoxA-*opp3*-3′ | ATTTTTTCTTCTTACCTGCCGATTTCTAAATTTCCATTCCCTTC |
| | BoxB-*opp3*-5′ | GGAAATTTAGAAATCGGCAGGTAAGAAGAAAAAATAATATGCTTTG |
| | BoxB-*opp3*-3′ | CGGGATCCGGTCCAGTAATAAATCCTGTTAAG |
| *opp-4* | BoxA-*opp4*-5′ | CGGGATCCGCAAGAGCACACTGGGACAAAGC |
| | BoxA-*opp4*-3′ | GCATCCATATCCACGGCCCCATACATATGCCTCCTACTTTC |
| | BoxB-*opp4*-5′ | AGGCATATGTATGGGGCCGTGGATATGGATGCCACCTGC |
| | BoxB-*opp4*-3′ | CGGGATCCCCTGCTGGTCTATTAACATATCCACC |
| *dtpT* | BoxA-*dtpT*-5′ | CGGGATCCAGCATCAAGGTCGTGACTATTTC |
| | BoxA-*dtpT*-3′ | GCGTTAGTTAAGTACCACCTTTCCCAGAACTCTACAAAG |
| | BoxB-*dtpT*-5′ | GAGTTCTGGGAAAGGTGGTACTTAACTAACGCTTCTGC |
| | BoxB-*dtpT*-3′ | CGGGATCCTGCAGAAATCATTTGTTCACTAGC |

[a] BamHI restriction sites are underlined.

human diseases ranging from superficial skin abscesses to serious infections such as pneumonia, endocarditis, and severe sepsis. This bacterium is one of the main agents of nosocomial and food-borne infections, suggesting its great potential for adaptation. Numerous studies have focused on the identification of virulence genes in *S. aureus*. However, no detailed analysis of the *opp* genes of *S. aureus* has been carried out. We initiated a systematic analysis of these transporters. Our results indicate that *S. aureus* encodes four distinct putative Opp systems, which differ from one another on the basis of their (i) genetic organization, (ii) amino acid sequence, (iii) regulation of expression, and (iv) physiological roles.

## MATERIALS AND METHODS

**Bioinformatic procedures.** DNA sequence analysis was performed from the genomes of the *S. aureus* strains JH1 and JH9 (unfinished sequences; accession numbers for JH1, NZ_AAPK00000000 and AAPK00000000; accession numbers for JH9, NZ_AAPL00000000 and AAPL00000000,), NCTC8325 (CP000253 and NC_007795), N315 (25), Mu50 (25), COL (17), MRSA252 (22), MSSA476 (22), MW2 (4), USA300 (13), and RF122 (AJ938182 and NC_007622). Percentages of identity between nucleotide or amino acid sequences were determined using the FASTA sequence comparison program (35) and the AliBee-multiple alignment method (33), respectively. Putative promoter sequences were identified using the BPROM prediction of bacterial promoter program (http://www.softberry.com). Putative terminators of transcription were determined using the RNA secondary structure prediction program (http://www.genebee.msu.su).

**Bacterial strain and growth conditions.** *S. aureus* RN6390 (36), a derivative of the clinical strain NCTC8325, was grown aerobically with shaking at 37°C in chemically defined medium (CDM) (45), supplemented with biotin (0.1 mg liter$^{-1}$) and calcium panthotenate (2 mg liter$^{-1}$). Cultures in CDM were performed either in complete CDM (CM), containing 18 amino acids in their free form, or in minimal CDM (MM) containing 9 amino acids (Glu, Leu, Cys, Met, Gly, Val, Thr, Arg, and Lys). MM medium, designed by studying RN6390 auxotrophy, ensured bacterial growth up to an optical density at 600 nm ($OD_{600}$) of 2. When required, MM was supplemented with 1% (wt/vol) pancreatic enzyme digest of casein (Bacto Tryptone; Difco Laboratories). Cultures in all CDMs were inoculated with cells grown overnight in CM and washed twice in 50 mM phosphate buffer (pH 6.8). For peptide utilization experiments, CM was depleted of the essential amino acid glutamic acid that was supplied in a peptide at a glutamic acid final concentration of 3.5 mM. All peptides were from a commercial source (Sigma, St. Louis, MO).

**Construction of peptide transport mutants in *S. aureus* RN6390.** Peptide transport mutants of *S. aureus* RN6390 were obtained by single or successive gene deletions. In a first step, a recombination cassette was cloned in *E. coli* using an overlap PCR technique (12, 30). Briefly, two fragments (called boxes A and B, corresponding to upstream and downstream regions of the fragment to be deleted, respectively) were amplified by PCR from RN6390 chromosomal DNA using external primers that incorporate terminal BamHI restriction sites and internal primers that contain 16 complementary nucleotides between boxes A and B (12, 30). Nucleotide primers used for PCR amplification are listed in Table 1. PCR products were purified by agarose gel electrophoresis, quantified, mixed at equal concentrations, and used as a template for a second round of PCR using only the two external primers (overlap PCR). The hybrid PCR product (joining boxes A and B and termed boxAB), which corresponds to the recombination cassette, was cloned into the pCR-Blunt II-TOPO vector (Invitrogen), resulting in plasmid pTOPO::boxAB. The BamHI-boxAB fragment was then purified and cloned into the temperature-sensitive shuttle vector pMAD (3). The resulting plasmid pMAD::boxAB was introduced into *S. aureus* RN4220 by electroporation, and erythromycin-resistant transformants were selected at 30°C, the permissive temperature for plasmid replication. *S. aureus* RN6390 was then transformed with pMAD::boxAB purified from RN4220 transformant. Deletion of the chromosomal region was subsequently obtained by double-crossover events as previously described (39). Chromosomal deletions were checked by PCR and Southern blotting of BclI-digested genomic DNA from mutant and wild-type strains with probes specific for boxes A or B.

**Production of anti-OppA antibodies.** The *opp-1A*, *opp-3A*, and *opp-4A* genes (deprived of signal sequence codons) were amplified by PCR from chromosomal DNA of *S. aureus* RN6390 using the following primers: *opp-1A*, 5′-CACCAATAAAGGTTTAGAGGAGAAAAAAG-3′ and 5′-TTATTTATACTGCATTTCATTGAATGG-3′; *opp-3A*, 5′-CACCAATGACGATGGTATTTATTCAGATAAAGG-3′ and 5′-TTATTTTTTCTTCTTACCTGTTTC-3′; *opp-4A*, 5′-CACCAGCAGTAATAAAGATGAAGGAGTAAAAG-3′ and 5′-CTAAGCTTCTTTAGTTAAATTATATAAAC-3′. Amplification products were cloned in *E. coli* BL21(DE3) (Invitrogen) using the bacterial expression vector pET100/D-TOPO (Invitrogen) containing a $His_6$ tag.

TABLE 2. Oligonucleotide primers used for RT-PCR

| Gene or region | Gene(s)[a] | First primer (5′–3′) | Second primer (5′–3′) |
|---|---|---|---|
| Transcriptional analysis | | | |
| *opp-1* region | SAOUHSC_02770–SAOUHSC_02768 | GCCACAAGCCCATCGTGTTG | CTTACCTTCCAGATGTTGATGCG |
| | SAOUHSC_02769–*opp-1A* | CAGCGGAAGATAAGTGGCAATGTG | CCAGTACCATCGAACTTTTTAACGCC |
| | *opp-1A*–*opp-1B* | GCCTCGTCCATATGTATTTGTGTCTCC | CCTGTTAATCCAGAAGTCGGC |
| | *opp-1B*–*opp-1F* | GATCGTGCGATACGTTCAGT | CACTTTCTTCTTATGCATCGGTTG |
| | *opp-1F*–SAOUHSC_02762 | GTGGTAGCGGTAAATCGACG | CTACTCGCTGGACGTGGTGTTGGC |
| *opp-2* region | SAOUHSC_01382–*opp-2B* | CGGCTATGAGGGCGCTGAACTTCGC | GCATGACGGTCACTACTTTCTG |
| | *opp-2B*–*opp-2F* | GTTTACCAGCGTTCTTTATCGG | CAGTATTATCAGTTAGACCTCTG |
| | *opp-2F*–*SAOUHSC_01376* | CCTGATTGGACGATTGAGACCTC | GCATCGCGGAGTTCTTCTTCACTCA |
| opp-3 region | *opp-3B*–*opp-3D* | TAGGTGTTGCAGCAGCTACTA | CTACGCCTCTCACTGCCTGC |
| | *opp-3D*–*opp-3F* | GAAGTAAATGATTTGCATGTTTCC | CCCCTTTGTATATATCAAACG |
| | *opp-3F*–*opp-3A* | ACGAAGTGAGAGCGATTGAA | GAGGTGCAAAGGTATTAAGTGC |
| *opp-4* region | *opp-4A*–*opp-4F* | CATTGAAATTGGAACTGATCAAAG | CAACAGCTTTAACGTACTGAG |
| | *opp-4F*–*opp-4B* | GCACATGAATTTTCAGGTGGAC | CCTAAAGTGAATGCAGGTAA |
| | *opp-4B*–*opp-4C* | CCCGTTTCAAGGTTCTGTTG | GAACAAGTCTTGCAATACCTCC |
| *opp-5A* | *opp-5A* | ATGCATTCATCTGGCAAAGACTTA | TTATCGTTCAATCGTTGTTCGATAATCG |
| *opp* expression analysis | | | |
| *opp-1A* | *opp-1A* | GCCTCGTCCATATGTATTTGTGTCTCC | CTGCTTGTAAGTATTCTGCTTGTTC |
| *opp-2B* | *opp-2B* | GTTTACCAGCGTTCTTTATCGC | GCATGACGGTCACTACTTTCT |
| *opp-3A* | *opp-3A* | GAATTAGAAAAGCCGGTTCCATATAT | GGCGAATTCATGGTACTCGCAT |
| *opp-4A* | *opp-4A* | CTTAACAGGATTTATTACTGGACC | CTTTTAGGATCTTCTCTAAATCCG |
| *opp-4D* | *opp-4D* | CCTCATCAATTATCTGGTGG | CAACAGCTTTAACGTACTGAG |
| *opp-5A* | *opp-5A* | CACCACTGTGACGTACCAAGAAGACGG | TTATCGTCAATCGTTGTTCGATAATC |

[a] For gene pairs, the PCR product begins in the first gene named and ends in the second.

Production of the Opp-1A, Opp-3A, and Opp-4A $His_6$-tagged proteins was carried out as follows. Cells were grown to an $OD_{600}$ of 0.5 in Luria-Bertani medium containing ampicillin (50 μg $ml^{-1}$) at 37°C. Expression of *oppA*-$His_6$ recombinant genes was induced for 2 h with isopropyl-β-D-thiogalactopyranoside (1 mM). Cells were harvested by centrifugation; resuspended ($OD_{600}$ of 50) in 50 mM $NaH_2PO_4$, 300 mM NaCl, and 10 mM imidazole; and broken using a cell disrupter system (Constant Systems Ltd). Cell debris was removed by centrifugation, and the supernatant was loaded on a His-select nickel affinity resin column (QIAGEN). The $His_6$-tagged proteins were eluted with an imidazole gradient (20 to 250 mM). Eluted fractions were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (26) and analyzed by Western blotting using monoclonal anti-polyhistidine antibody (Sigma, St. Louis, MO). The OppA-$His_6$ containing fractions were dialyzed against 50 mM $NaH_2PO_4$ and 300 mM NaCl and used for custom antibody production in guinea pigs (Centre de Production Animale, Olivet, France). Antisera were tested for their specificity and efficiency using known amounts of purified OppA-$His_6$ proteins. All were highly specific and produced signals with comparable intensities when used at the same dilution.

**Preparation of *S. aureus* protein extracts and Western blot analyses.** Total cell protein extracts were prepared essentially as described previously (39). Briefly, *S. aureus* cells were harvested by centrifugation, precipitated with 10% (vol/vol) trichloroacetic acid, washed in 80% (vol/vol) acetone, incubated for 90 min at 37°C in the presence of lysostaphin (100 μg $ml^{-1}$), and lysed with 2% (vol/vol) SDS. Protein concentration was evaluated using a detergent-compatible assay (Bio-Rad). For each sample, 20 μg of total cell protein extract was loaded and separated on SDS-polyacrylamide gel electrophoresis gels and electrotransferred on Immobilon-FL transfer membrane (Millipore). Opp-1A, Opp-3A, and Opp-4A were detected using corresponding polyclonal guinea pig antibodies (dilution, 1:10,000). Immunodetection was carried out with Alexa Fluor 488 anti-guinea pig immunoglobulin G (Invitrogen), followed by visualization (at 540 nm) using a Fluorimager 595 (Amersham Biosciences). Fluorescence signal was quantified using the Image Quant program (version 5.2; Molecular Dynamics). Each experiment was performed three times with independent protein extracts.

**RNA isolation and RT-PCR amplification.** Total RNA was extracted from *S. aureus* RN6390 grown in CDM. Cells were sedimented by centrifugation and resuspended in 500 μl of cold TE buffer (10 mM Tris, 1 mM EDTA, pH 8). Cell suspension was transferred to a microcentrifuge tube containing 0.5 g of glass beads (0.1 mm in diameter), 30 μl of 3 M sodium acetate (pH 5.2), 500 μl of water-saturated phenol-chloroform (5:1), and 30 μl of 10% (vol/vol) SDS. Cells were disrupted using a Fast Prep FP120 system (Bio101 Thermo Electron Corp.). After removal of nonsoluble material (centrifugation at 13,000 × *g* for 15 min at 4°C), RNA was extracted with 1 volume of phenol-chloroform (5:1) and washed in 1 volume of chloroform. Total RNA was purified using a High Pure RNA Isolation kit (Roche). RNA concentration was evaluated by measuring the $OD_{260}$. Extracts were adjusted to 0.5 μg $\mu l^{-1}$ and tested for DNA contamination by PCR prior to reverse transcription (RT). Annealing of 2 μg of total RNA with random nonamers (CyScribe cDNA Post labeling Kit; Amersham Biosciences) was performed for 10 min at 20°C after denaturation of RNA secondary structures (5 min at 70°C). cDNA synthesis by RT was accomplished at 42°C for 2 h with PowerScript Reverse Transcriptase and Ultrapure deoxynucleoside triphosphate mix (Clontech/Takara Bioscience), followed by enzyme inactivation (15 min at 70°C). For limit dilution (LD) RT-PCR, serial dilutions (1:1, 1:10, 1:100, 1:500, and 1:1000) of cDNA were performed, followed by amplification of each dilution by PCR using the oligonucleotides listed in Table 2. PCR (30 cycles) was performed as follows: 94°C for 30 s, 50°C for 30 s, and 72°C for 1 to 3 min using a *Taq* Core Kit (Qbiogene). Transcripts of the *hu* gene were used as internal controls (15). Results were quantified when needed using Image Quant software. Each experiment was performed three times.

**RP-HPLC analyses.** Peptide consumption during growth of RN6390 and mutant derivatives was done in MM supplemented with peptides and was evaluated by reverse-phase high-performance liquid chromatography (RP-HPLC). Cells were removed from stationary phase culture medium by centrifugation. Supernatant was supplemented with trifluoroacetic acid (1%, vol/vol), centrifuged, and filtered through 0.22-μm-pore-size filters (Millipore). Peptide separation was carried out at 40°C using an RP18 X-Terra column (250 by 4.6 mm; Waters) at a flow rate of 1 ml $min^{-1}$. Solvent A was 0.115% (vol/vol) trifluoroacetic, and solvent B was 0.1% (vol/vol) trifluoroacetic acid plus 60% (vol/vol) acetonitrile in MilliQ water. A 5-min isocratic phase in solvent A was followed by a linear gradient of solvent B (0 to 60% within 40 min). Eluted peptides were detected by UV detection at the $OD_{214}$.

## RESULTS

***S. aureus* harbors four distinct putative oligopeptide permease systems.** Computational analysis of the 11 available genomic DNA sequences of *S. aureus* strains revealed the presence of four distinct putative *opp* operons (Fig. 1). The *opp-1ABCDF* (genes SAOUHSC_02767 to SAOUHSC_02763) and *opp-2BCDF* (SAOUHSC_01380 to SAOUHSC_01377) operons have already been identified in *S. aureus* RN6390, a

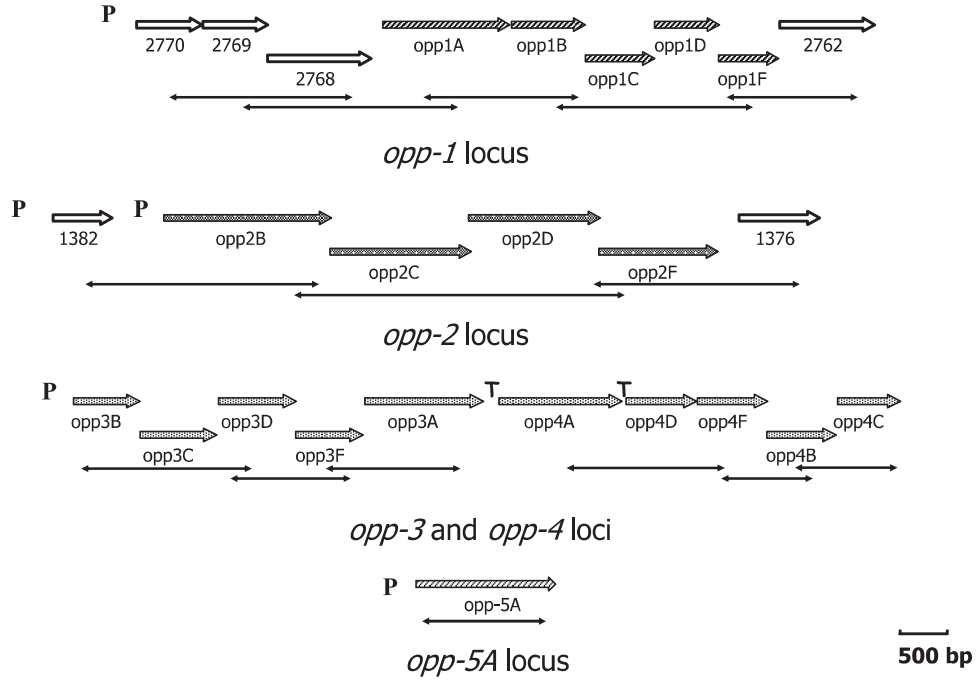


FIG. 1. Genetic and transcriptional organization of the *S. aureus* NCTC8325 *opp* systems. The *opp* genes were designated according to their homology with characterized bacterial *opp* genes. Gene numbers were adapted from the genomic DNA sequence annotation of *S. aureus* NCTC8325 (accession numbers CP000253 and NC_007795). Open reading frames adjacent to the represented loci are present on the opposite strand. Double arrows indicate the amplification products obtained during RT-PCR experiments (see Results). Primers for transcriptional analysis are listed in Table 2. P, putative promoter sequence; T, putative terminator of transcription.

derivative of the NCTC8325 sequenced strain (9). By sequence homology searches using the *opp-1* sequence of NCTC8325, we found two additional adjacent *opp* operons, *opp-3* (genes SAOUHSC_00923 to SAOUHSC_00927) organized as *oppBCDFA*, and *opp-4* (genes SAOUHSC_00928 to SAOUHSC_00932) organized as *oppADFBC* and located 212 bp downstream of *opp-3*. An additional isolated *oppA*-like gene (gene SAOUHSC_00201) encoding a putative oligopeptide binding protein, Opp-5A, was also identified in all strains. Organization of these *opp* systems is poorly conserved from one system to another. This is also the case for their amino acid sequence. For example, four putative OppA proteins of NCTC8325 share about 28% identity (Table 3), while transmembrane proteins, or ATPase proteins, each share between 27 and 35% identity. All the OppA proteins carry the typical signature of bacterial extracellular solute-binding proteins, and all OppD and OppF proteins carry the ABC transporter signature sequence (42, 44).

Each *opp* cluster is highly conserved between strains of *S. aureus*, with identity in most cases greater than 99% at the nucleotide level. One exception is *opp-4*, which is interrupted by a transposase gene in *opp-4A* of *S. aureus* MRSA252 (the most genetically diverse human strain) and absent from strain RF122 (isolated from bovine mastitis). Surprisingly, the RF122 genome appears to have a duplication of the *opp-3* cluster: an *opp* cluster located 169 nucleotides downstream of *opp-3* displays the same genetic organization and encodes proteins closely related to Opp-3 proteins. Nevertheless, two stop codons are present in the *oppA* gene of this additional *opp* cluster, leading to a nonfunctional OppA protein. Finally, one of the 11 sequenced strains of *S. aureus*, USA300 (a highly virulent isolate), contains a fifth *oppABCDF* operon in a novel mobile genetic element termed ACME that is absent from other sequenced *S. aureus* strains (13).

TABLE 3. Identity (%) between the OppA proteins of *S. aureus* NCTC8325

| Protein | % Identity with the indicated protein of strain NCTC8325 | | | |
|---|---|---|---|---|
| | Opp-1A | Opp-3A | Opp-4A | Opp-5A |
| Opp-1A | | 27.5 | 29 | 26 |
| Opp-3A | | | 28.5 | 30 |
| Opp-4A | | | | 29 |

A search for homologs of *S. aureus* Opp proteins revealed that *opp-1*, *opp-2*, *opp-3*, and *opp-5A* are highly conserved in *Staphylococcus epidermidis*, the second clinically important staphylococcal species (Table 4). Moreover, Opp-3 is also detected in all other sequenced staphylococcal species, namely *Staphylococcus haemolyticus* and *Staphylococcus saprophyticus*. Interestingly, Opp systems that have been shown to be implicated in bacterial nutrition in *L. lactis*, *L. monocytogenes*, and *Streptococcus agalactiae* are most similar to the *S. aureus* Opp-3 system (6, 27, 40) (Table 4).

**All Opp systems are expressed during growth in CDM.** Expression of the different *opp* genes of *S. aureus* RN6390 was analyzed in exponential MM cultures by semiquantitative RT-PCR using specific *opp* primers. All *oppA* genes were expressed (Fig. 2A), and similar results were also obtained for the expression of other *opp* genes (data not shown). Their expression

TABLE 4. Identity between Opp proteins of *S. aureus* NCTC8325 and those from other gram-positive bacteria

| *S. aureus* Opp protein[a] | % Identity with protein from: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *S. epidermidis* | *S. haemolyticus* | *S. saprophyticus* | *B. halodurans*[b] | *B. thuringiensis* | *L. monocytogenes* | *S. agalactiae* | *L. lactis*[c] |
| 1A | 82 | | | 54 | | | | |
| 1B/C | 80 | | | 54 | | | | |
| 1D/F | 68 | | | 47 | | | | |
| 2B/C | 68 | | | | | | | |
| 2D/F | 57 | | | | | | | |
| 3A | 66 | 68 | 61 | | 42 | 40 | 39 | 35 |
| 3B/C | 87 | 89 | 85 | | 58 | 54 | 51 | 45 |
| 3D/F | 85 | 88 | 86 | | 66 | 65 | 66 | 59 |
| 4A | | | | 32 | | | | |
| 4B/C | | | | 42 | | | | |
| 4D/F | | | | 53 | | | | |
| 5A | 57 | | 62 | | | | | |

[a] Values indicated for OppB/C and OppD/F correspond to the means of the percent identities of the transmembrane (OppB and OppC) and the ATP-binding (OppD and OppF) proteins. Identities of <30 % were not considered.
[b] *B. halodurans*, *Bacillus halodurans*.
[c] Values are for percent identities between Opp-3 proteins of *S. aureus* and Opt proteins of *L. lactis*.

levels depended on the *opp* system under study. Under our experimental conditions and assuming that messenger stabilities are comparable, the *opp-3A* gene was apparently the most highly expressed *oppA*. To verify these observations, we evaluated the production of the three binding proteins Opp-1A, Opp-3A, and Opp-4A by Western-blot analyses using specific antibodies. The expression level depended on the protein (Fig. 2B), with a predominant production of Opp-3A. These results correlated with the observed levels of *opp-1A*, *opp-3A*, and *opp-4A* transcripts.

We performed further RT-PCR transcriptional analysis on total RNA extracted from *S. aureus* RN6390 cells grown in MM to examine operon expression (Fig. 1). Results indicated that genes SAOUHSC_02770 to SAOUHSC_02762, comprising the *opp-1* genes, are organized in an operon and are transcribed polycistronically. This would be expected from examination of the *opp-1ABCDF* cluster in *S. aureus* NCTC8325 as (i) transcriptional signals upstream and downstream of *opp* genes were not found, and (ii) codirectional ORFs are present upstream and downstream of the *opp* genes (Fig. 1). According to *S. aureus* NCTC8325 genome annotation, SAOUHSC_02770 was referenced as the diaminopimelate epimerase DapF, SAOUHSC_02769 as the Zn-dependent alcohol deshydrogenase AdhP, SAOUHSC_02768 as a conserved hypothetical protein of unknown function, and SAOUHSC_02762 as AraJ, an arabinose efflux permease.

Transcriptional coupling of SAOUHSC_01376 and *opp-2BCDF* was consistent with the absence of a transcriptional terminator downstream of *opp-2F* (Fig. 1). Cotranscription of SAOUHSC_01382 with *opp-2BCDF*, despite a putative promoter sequence upstream of *opp-2B*, presumably results from the absence of a terminator downstream of SAOUHSC_01382.

The *opp-3BCDFA* cluster contained a putative promoter sequence in front of *opp-3B* and a transcriptional terminator downstream of *opp-3A* ($-14.6$ kcal mol$^{-1}$). These features are in agreement with a polycistronic transcription of the *opp-3* genes and the lack of evidence for cotranscription of *opp-3* and *opp-4* (no RT-PCR product could be obtained between *opp3A* and *opp4A* genes).

In the case of *opp-4*, RT-PCR results indicated transcriptional coupling of *opp-4ADFBC*, despite the presence of a terminator structure in the noncoding region between *opp-4A* and *opp-4D* ($-16.8$ kcal mol$^{-1}$). No consensus promoter was

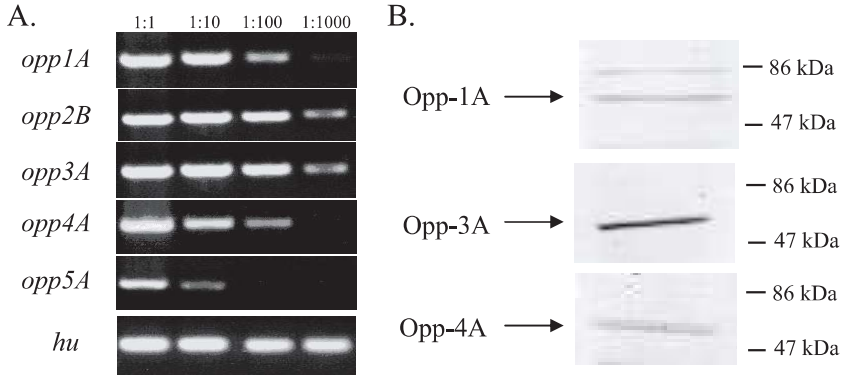


FIG. 2. Expression of *opp* genes and production of OppA proteins in *S. aureus* RN6390 grown in MM. Proteins and RNA were extracted in exponential growth phase ($OD_{600}$ of 0.5). (A) *opp* transcription analyzed by RT-PCR. cDNA dilutions used for transcript quantification were 1:1, 1:10, 1:100, and 1:1,000. *hu* transcript was used as an internal standard. (B) OppA production analyzed by Western blotting. Opp-1A, Opp-3A, and Opp-4A proteins were detected at their expected sizes of 58, 59, and 62 kDa, respectively.

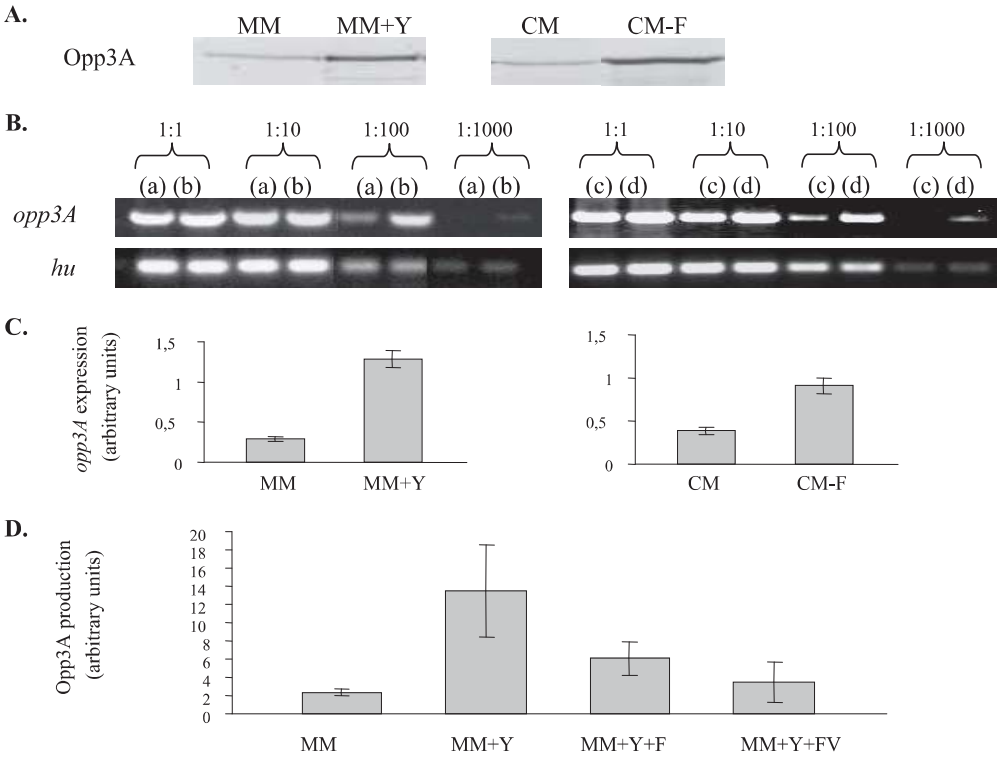


FIG. 3. Regulation of Opp-3A by the amino acid content of the medium. Proteins and RNA were extracted in exponential phase of growth ($OD_{600}$ of 0.5). (A) Opp-3A production analyzed by Western blotting. Opp-3A was detected at the expected size of 59 kDa. MM+Y, MM supplemented with Tyr; CM-F, CM depleted of Phe. (B) *opp-3A* transcription analyzed by RT-PCR. cDNA dilutions used for transcript quantification were 1:1, 1:10, 1:100, and 1:1,000. *hu* transcript was used as an internal standard. a, MM; b, MM+Y; c, CM; d, CM-F. (C) Quantification of *opp-3A* transcripts. Values are the means of three independent experiments with standard deviations. Transcripts were quantified using the 1:100 dilution samples. For each experiment, the amount of *opp-3A* transcript was given as a ratio to that of *hu*. (D) Quantification of Opp-3A production. Proteins were extracted in exponential growth phase in MM ($OD_{600}$ of 0.5). Values are the means of five independent determinations with standard deviations. MM+Y, MM supplemented with Tyr; MM+Y+F, MM supplemented with Tyr and Phe; MM+Y+FV, MM supplemented with Tyr and the dipeptide Phe-Val.

predicted in front of *opp-4A*, which might explain the weak expression levels for *opp-4A*.

No transcriptional terminators were detected in the noncoding regions downstream of clusters containing *opp-1*, *opp-2*, *opp-4*, and *opp-5A*. However, downstream open reading frames were all divergently transcribed, thus circumscribing the *opp* transcriptional units.

**Expression of *opp-3* but not other operons is modulated by aromatic amino acids.** Previous results revealed that the *S. aureus opp* operons were not equally expressed during growth in CDM, suggesting specific transcriptional regulatory mechanisms. In several bacteria, *opp* expression is known to be regulated by nitrogen sources, including specific amino acids (1, 2, 20). Therefore, the possible effect of free amino acids on production of *S. aureus* Opp transporters was investigated. Two complementary approaches were developed. Oligopeptide-binding protein production was estimated by Western blot experiments in exponential phase cultures, either in MM supplemented with one of the nine nonessential amino acids (Pro, Ala, Ile, Phe, Trp, Tyr, Ser, Asn, and His) or in CM depleted of one of these amino acids. The external amino acid pool affected the protein production of Opp-3A only: addition of Tyr to MM and removal of Phe from CM significantly ($P < 0.001$) increased (about sixfold) Opp-3A production (Fig. 3A).

To ascertain the effects of the amino acids Tyr and Phe, a semiquantitative transcriptional analysis (LD RT-PCR) was performed on all *opp* systems using the primers listed in Table 2. RNA was extracted from mid-exponential cultures in MM supplemented or not with Tyr and in CM depleted or not of Phe. Only expression of *opp-3* was affected by the presence of Tyr in MM or by the removal of Phe from CM (Fig. 3B). Amounts of *opp-3A* mRNA were ~5-fold higher in cells grown in Tyr-supplemented MM than in MM and ~2.5-fold higher in Phe-deprived CM than in CM (Fig. 3C). Similar results were obtained using *opp-3C* primers (data not shown), in agreement with the cotranscription of the *opp-3BCDFA* genes.

These results indicate that Tyr and Phe exert opposite effects on *opp-3* expression. Surprisingly, addition of Phe to MM did not affect the production of Opp-3A (data not shown). A possible explanation is that only Tyr affects *opp-3* expression, whereas Phe acts as a competitor, preventing the action of Tyr. To evaluate this hypothesis, we compared Opp-3A production in MM supplemented with Tyr or with both Tyr and Phe. In the presence of Phe, stimulation of Opp-3A production by Tyr was significantly reduced (Fig. 3D), which therefore supports the hypothesis of a competitive effect of Phe on Tyr induction. Phe might impair Tyr entry into the cell (competition for transport by the same amino acid permease) or prevent its binding to an

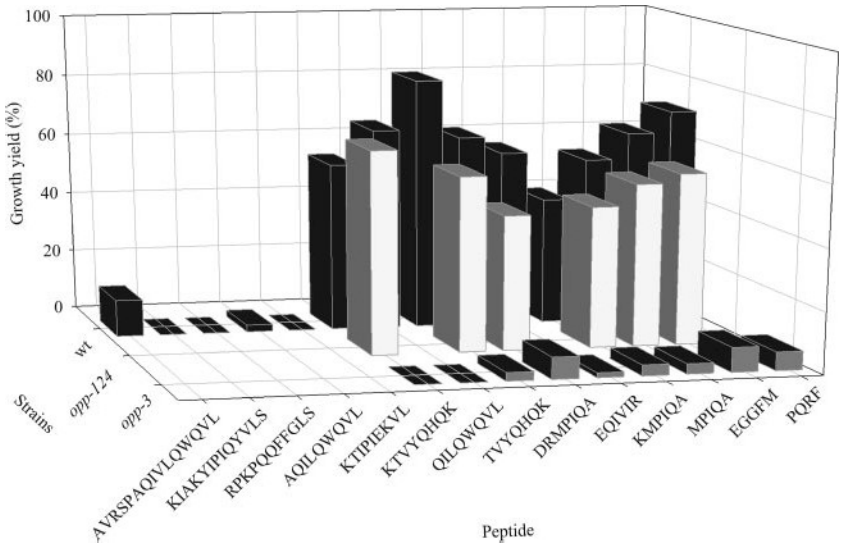


FIG. 4. Peptide utilization by wild-type and *opp* mutant strains of *S. aureus*. Growth yield was calculated as the percentage of population (final OD) reached in peptide-containing medium compared to CM in the stationary phase of growth. QILQWQWL, EQVIR, and PQRF peptides were not tested on the *opp-1 opp-2 opp-4* (*opp-124*) mutant strain. wt, wild type.

intracellular target. To further discriminate between these hypotheses, Opp-3A production was compared in MM containing Tyr and supplemented with Phe brought either in its free form or in a dipeptide form. The inhibitory effect of Phe was in the same range under either condition (Fig. 3D), thus favoring the hypothesis that Phe is likely to compete with Tyr for an intracellular target.

Altogether, these results indicate that *opp-3* operon transcription is influenced by the aromatic amino acids Tyr and Phe and suggest that the two amino acids might compete for an intracellular target.

**Depletion of Opp-3, but not other Opp proteins, prevents growth of *S. aureus* in peptide-containing CDM.** As all Opp systems were expressed during growth in CDM, this medium was suitable to evaluate involvement of Opp systems in nitrogen nutrition. This was estimated by replacing an essential amino acid by a peptide containing the amino acid. As expected, removal of Glu and Gln from the complete CDM prevented growth of *S. aureus* RN6390. Glu (or Gln)-containing peptides from 2 to 8 amino acid residues were able to sustain growth. In contrast, no growth could be detected when medium was supplemented with peptides containing 9 amino acid residues or more (Fig. 4). These results suggest that (i) at least one of the Opp systems was able to fulfill the amino acid requirement of the strain and (ii) *S. aureus* was unable to use peptides larger than octapeptides for nutrient purposes.

To determine which of the systems is involved in nutrient uptake, the different *opp* operons were deleted by double-crossover events, resulting in the *opp-1*, *opp-2*, *opp-3*, and *opp-4* mutant strains. Combinations of deletions were also constructed, and the *opp-1 opp-2 opp-4* triple mutant strain was also obtained. Growth of each mutant was similar to that of the parental strain in complete CDM (data not shown). Deletion of *opp-1*, *opp-2*, or *opp-4* alone or in combination did not affect the ability of *S. aureus* RN6390 to use tested peptides as nutrients, regardless of the peptide length. In contrast, deletion of *opp-3* fully impaired the use of 4-mer to 8-mer peptides as a source of Glu/Gln (Fig. 4).

Interestingly, all dipeptides and most tripeptides were able to sustain growth of the *opp* mutants, regardless of the inactivated system. As a di- or tripeptide permease-like gene (*dtpT*) is present in all *S. aureus* genomes (SAOUHSC_00738 of *S. aureus* NCTC8325), this transporter is very likely involved in di- and tripeptide uptake. To evaluate the respective roles of DtpT and Opp in nutrient di- and tripeptide utilization, *dtpT*, *opp-3 dtpT*, *opp-1 opp-2 opp-4 dtpT*, and *opp-1 opp-2 opp-3 opp-4 dtpT* mutant strains were constructed, and their ability to utilize di- or tripeptides for growth was evaluated. The mutant strain *dtpT* did not grow when Glu/Gln was provided in a dipeptide form, indicating that DtpT only is responsible for the use of dipeptides (Fig. 5). Of the four tripeptides tested, Pro-His-Glu was imported exclusively by Opp-3, Lys-Glu-Gly and Gly-Gly-Gln were used by DtpT only, and Ser-Glu-Gly was imported by both systems, as only the double deletion *opp-3 dtpT* impaired the growth (Fig. 5).

To complete this study with a larger range of peptides, *dtpT* and combined *opp dtpT* mutant strains were grown in MM supplemented with a pancreatic digest of caseins containing a large mix of peptides. All strains grew equally well, as the casein digest also contained a large amount of free amino acids. A control RT-PCR experiment indicated that the four *opp* systems of the wild-type strain were expressed (data not shown). The peptide content of the medium from stationary phase cultures was analyzed by RP-HPLC. No difference in peptide content was detectable between medium from the *dtpT* mutant and *opp-1 opp-2 opp-4 dtpT* mutant strains (data not shown). Chromatograms could be superimposed, suggesting that the peptide contents were qualitatively and quantitatively comparable. In contrast, several peaks were detected in growth medium of the *opp-3 dtpT* mutant (Fig. 6). Moreover, some of the common peaks were present in larger amounts in the *opp-3*

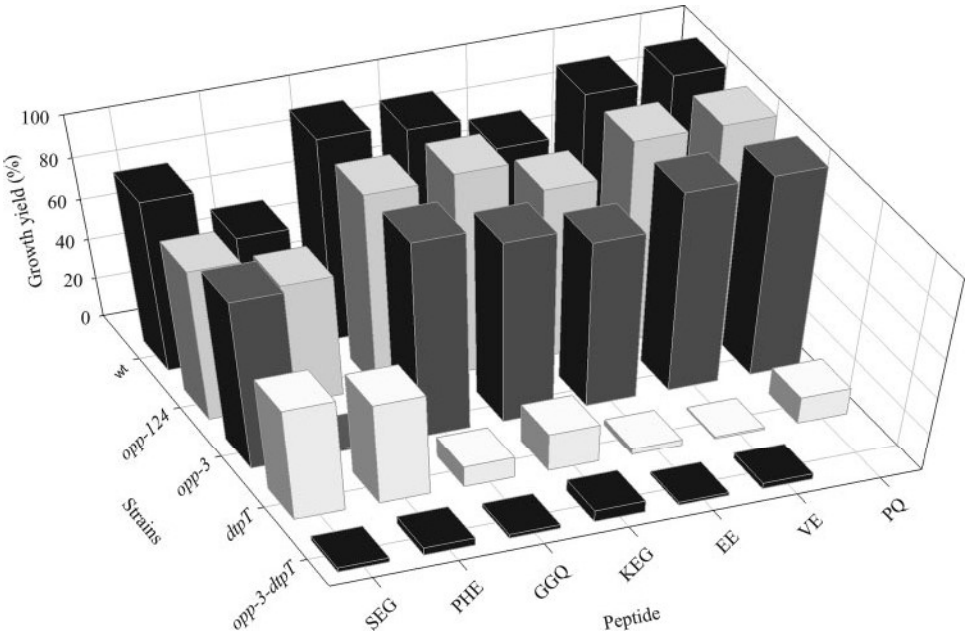


FIG. 5. Di- and tripeptide utilization by *opp-3* and *dtpT* mutants of *S. aureus*. Growth yield was calculated as the percentage of population (final OD) reached in peptide-containing medium compared to CM in the stationary phase of growth. wt, wild type.

*dtpT* culture medium. This situation most probably corresponded to coeluting peptides, of which one accumulated with the *opp-3 dtpT* mutant only. Similar profiles were obtained with the *opp-1 opp-2 opp-3 opp-4 dtpT* mutant strain (data not shown). Altogether, these results indicate a prevalent role of Opp-3 in oligopeptide utilization during growth of *S. aureus* RN6390. Among the 4 putative Opp systems of *S. aureus*, only Opp3 is involved in nutrition, transporting efficiently tri- to octapeptides with overlapping substrate specificity with DtpT concerning some tripeptides.

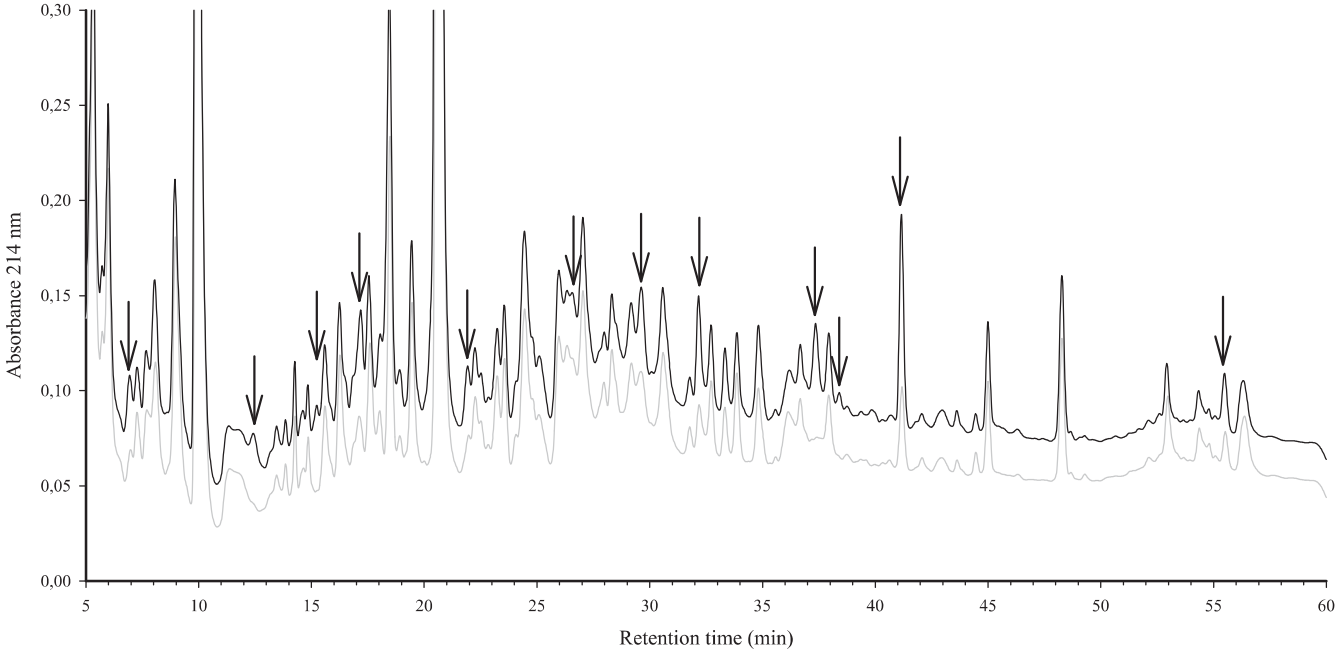


FIG. 6. Peptide content of the growth medium of *S. aureus dtpT* and *opp-3 dtpT* mutants. Cells were grown in CM supplemented with casein-derived peptides to the stationary phase, and peptide content of the external medium was analyzed by RP-HPLC. From bottom to top, the strains are the *dtpT* and *opp-3 dtpT* mutants. Arrows highlight main peaks that were detected at higher levels or only in the culture medium of the *opp-3 dtpT* mutant.

## DISCUSSION

Genome analysis of 11 *S. aureus* strains revealed the presence of three putative complete Opp systems, Opp-1, Opp-3, and Opp-4, of which two, Opp-3 and Opp-4, are described for the first time. Each of them comprises a peptide-binding protein, two integral membrane proteins, and two ATPases. They all have the signatures of oligopeptide/dipeptide/nickel transport systems. In addition, the 11 sequenced *S. aureus* strains express a particular *opp* operon, *opp-2BCDF*, which lacks the substrate-binding protein. This system could act as an exporter, as export systems usually do not require a substrate-binding component needed for uptake (21). However, as Opp-2 is classified as an importer in the ABCISSE database (41), it is also possible that it recruits one of the four OppA proteins for peptide binding. Several Opp systems recruit peptide-binding protein(s) encoded by isolated genes located in another region of the genome (16, 34). In the case of Opp-2, a candidate partner is encoded by the orphan gene *opp-5A*. This suggestion is not supported by previous studies, as (i) in other cases, at least one *oppA* copy was also present within the operon, which is not observed with *S. aureus opp-2*; (ii) with the exception of the closely related species *S. epidermidis*, the unusual structure of the *opp-2* operon in *S. aureus* has not been reported in other bacterial genomes; and (iii) isolated peptide-binding proteins utilizing an Opp complex share significant homologies with the OppA protein encoded by the *opp* operon (16, 34). This is clearly not the case for Opp-5A, which revealed weak homologies with Opp-1A, Opp-3A, and Opp-4A (26, 30, and 29% identity, respectively). The roles of the atypical Opp-2 system and Opp-5A binding-protein thus remain to be determined.

Within a given strain, the *opp* operons display specific genetic organization and low degrees of sequence similarity. In contrast, each of the *opp* operons is highly conserved in all *S. aureus* strains, with the exception of *opp-4*, which is absent in RF122, altered in MRSA252, and missing from the *S. epidermidis* genome. These features and the low expression level of *opp-4* observed under different conditions suggest that this system might be involved in biological processes specific to certain staphylococci.

*S. aureus* is predicted to possess four distinct potential Opp systems. This multiplicity raises questions as to whether they have overlapping or distinct functions. One of the demonstrated roles of bacterial Opp systems is to provide peptides as nutrients. This study demonstrates that Opp-3 only is responsible for this nutritional function. Interestingly, Opp-3 is closer to bacterial Opp permeases involved in nutrient uptake than the other staphylococcal Opp systems (Table 1). The best documented nutritional oligopeptide transport system is by far the lactococcal Opp system. It transports peptides from 4 to up to 35 residues with little amino acid sequence specificity (14). Despite a more restricted capability in terms of peptide length (from 3 to 8 residues), the Opt system of *L. lactis* also seems to be characterized by broad substrate specificity (27), a feature that seems to be common to Opp systems involved in bacterial nutrition. From the results presented here, Opp-3 from *S. aureus* is capable of using peptides comprising 3 to 8 amino acid residues having unrelated sequence or biochemical features, which is in agreement with its nutritional function. In terms of preferences for peptide utilization, the staphylococcal Opp-3 system appears closer to Opt from *L. lactis* or Opp from *S. agalactiae* than to Opp or Ami from *L. lactis* and *S. thermophilus*, respectively (14, 16, 27, 40). These results are in concordance with sequence similarities (Table 4).

Opp-3 is the sole Opp system of *S. aureus* ensuring a nutritional function under the tested conditions. It is also the only staphylococcal Opp system whose expression was modulated by amino acids. In bacteria, nitrogen sources are known to regulate *opp* expression (31). For instance, *E. coli* K-12 *opp* expression is regulated by leucine, via the global regulator Lrp (8). Similarly, another global regulator, CodY, represses *L. lactis opp* expression in response to the intracellular pool of branched-chain amino acids (20). Nevertheless, regulation of *opp* genes by aromatic amino acids (as is the case for *S. aureus opp-3*) was not previously described. Aromatic amino acid regulation was previously demonstrated for other genes. The best documented case is the *tyrR* regulon of *E. coli* (37). TyrR is a transcriptional regulator that interacts with aromatic amino acids and binds to a specific DNA sequence. No TyrR homologue was found in the *S. aureus* genomes, thus making a regulatory pathway via TyrR unlikely in *S. aureus*. However, by using the dedicated program iMoMi (for interactive motif mining) (38), a putative regulatory binding motif was detected. It consisted of a 14-nucleotide repeated sequence located at −219 and −236 nucleotides upstream of the *opp-3B* start codon. This putative regulatory sequence was detected upstream of only one other gene among all available *S. aureus* genomes. The gene, SAOUHSC_02729 of *S. aureus* NCTC8325, encodes a putative amino acid transporter whose expression was also induced by Tyr, as revealed by LD RT-PCR analyses (data not shown). These preliminary data suggest a regulatory network implicating aromatic amino acids, oligopeptides, and amino acid transporter(s).

What are the roles of the other Opp systems? Opp-1, Opp-2, and Opp-4 are not involved in nutrient supply of *S. aureus*, at least under our experimental conditions. We cannot exclude the possibility that they can import nutritional peptides under different conditions, especially in the case of Opp-4, which was weakly expressed under all conditions tested. Alternatively, some of them could also be involved in the uptake of substrates other than peptides. Interestingly, Opp-1A is 35% identical to NikA from *E. coli* which has been experimentally shown to transport nickel (11, 32), whereas similarities to confirmed oligopeptide-binding proteins are lower (e.g., 22% with AmiA3 from *S. thermophilus* and 24% with OppA from *L. lactis*). *S. aureus* Opp-1A is also 39% identical to Gbs1577 of *S. agalactiae*, a binding protein of a putative peptide transporter that appeared not to be required for nitrogen nutrition and revealed similarity with $Ni^{2+}$ permeases (40). Transcriptional analysis of the *opp-1* region showed that additional genes were coexpressed with the *opp-1* genes, in correlation with their genomic organization. The corresponding proteins could be linked to the Opp-1 function. One of them (SAOUHSC_02770) encodes a protein homologous to DapF, a central enzyme in the biosynthesis pathways of both lysine and cell wall peptidoglycan in some bacterial species (23, 47). This could make sense as the *E. coli* Opp system has been shown to be involved in peptidoglycan turnover (34). Peptide uptake systems are also involved in other cellular functions, and the Opp-1 and Opp-2 systems have been suggested to play a role in virulence

in different animal models (5, 9, 29). Nevertheless, the exact contribution of these systems in the course of infection is unknown and remains to be elucidated.

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