Generic and Specific Adaptive Responses of *Streptococcus pneumoniae* to Challenge with Three Distinct Antimicrobial Peptides, Bacitracin, LL-37, and Nisin

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To investigate the response of *Streptococcus pneumoniae* to three distinct antimicrobial peptides (AMPs), bacitracin, nisin, and LL-37, transcriptome analysis of challenged bacteria was performed. Only a limited number of genes were found to be up- or downregulated in all cases. Several of these common highly induced genes were chosen for further analysis, i.e., *SP0385-SP0387* (*SP0385-0387* herein), *SP0912-0913*, *SP0785-0787*, *SP1714-1715*, and the *blp* gene cluster. Deletion of these genes in combination with MIC determinations showed that several putative transporters, i.e., *SP0785-0787* and *SP0912-0913*, were indeed involved in resistance to lincomycin and LL-37 and to bacitracin, nisin, and lincomycin, respectively. Mutation of the *blp* bacteriocin immunity genes resulted in an increased sensitivity to LL-37. Interestingly, a putative ABC transporter (SPI1715) protected against bacitracin and Hoechst 33342 but conferred sensitivity to LL-37. A GntR-like regulator, SP1714, was identified as a negative regulator of itself and two of the putative transporters. In conclusion, we show that resistance to three different AMPs in *S. pneumoniae* is mediated by several putative ABC transporters, some of which have not been associated with antimicrobial resistance in this organism before. In addition, a GntR-like regulator that regulates two of these transporters was identified. Our findings extend the understanding of defense mechanisms of this important human pathogen against antimicrobial compounds and point toward novel proteins, i.e., putative ABC transporters, which can be used as targets for the development of new antimicrobials.

Increased resistance of bacteria to commonly used antibiotics creates severe problems in treating infectious diseases. The resistance of one of the most important human pathogens, *Streptococcus pneumoniae*, to commonly used antibiotics has increased significantly in recent decades (15). This bacterium colonizes the nasopharynx and the upper respiratory tract asymptomatically. Nevertheless, under certain circumstances, *S. pneumoniae* can cause otitis media, meningitis, pneumonia, and sepsis (49). To cause disease, *S. pneumoniae* has to successfully colonize the mucosal surface of the nasopharynx, followed by dissemination to other parts of the human body. Mucosal surfaces of the human body form the first barrier that protects against pathogens. In this layer, mainly neutrophils and epithelial cells produce antimicrobial peptides (AMPs). Generally, AMPs display a cationic and an amphipathic nature, but they are variable in sequence, secondary structure, size, and mode of action (56). Antimicrobial peptides play an essential role in the host’s innate immune response (32).

One human AMP, the 18-kDa human cathelicidin antimicrobial protein hCAP-18 (16), is produced as an inactive proprotein that consists of a precursor protein, cathelin, and a carboxy-terminal peptide, LL-37 (64). LL-37 is a linear, 37-amino-acid-long cationic peptide with activity against Gram-positive and Gram-negative bacteria (76). It has been shown that the bactericidal action of LL-37 is due to immobilization of the peptide within the membrane lipid bilayer, where, as a consequence, it causes destabilization of the bacterial membrane (53).

In addition to coping with the human immune system, *S. pneumoniae* has to compete with other bacterial inhabitants, which also produce AMPs as a defense against competitors, to achieve successful colonization of the nasopharynx. AMPs generated by Gram-positive bacteria are named bacteriocins, and one of the best-characterized ones is nisin, produced by *Lactococcus lactis* and commonly used as a food preservative (59). The antimicrobial activity of nisin is rather broad against Gram-positive bacteria (17, 48). Nisin is able to inhibit peptidoglycan biosynthesis by interaction with lipid II and forms pores in bacterial membranes, which leads to cell death (7, 8, 21). Another attack and defense system used by bacteria is the production of antibiotics such as bacitracin. This toxic compound is a mixture of cyclic polypeptides produced by *Bacillus licheniformis* (28). Bacitracin is a nonribosomally synthesized antibiotic which, in Gram-positive cocci and bacilli, blocks biosynthesis of the bacterial cell wall by interaction with C55-isoprenyl pyrophosphate (2, 25, 66).

To establish whether *S. pneumoniae* contains general de-
fense mechanisms against heterologous AMPs, transcriptome analysis of *S. pneumoniae* D39 was performed upon challenge with three different antimicrobial peptides, i.e., LL-37, nisin, and bacitracin. The transcript levels of genes involved in various processes, such as gene regulation, transport, virulence, fatty acid synthesis, and phosphotransferase systems, had changed significantly. Several highly induced genes were chosen for further analysis. We show, for the first time to our knowledge, that some of these genes, encoding putative ABC transporters, are involved in the defense of *S. pneumoniae* against multiple antimicrobial compounds, e.g., bacitracin, nisin, LL-37, lincomycin, or Hoechst 33342. Furthermore, we demonstrate that the putative regulatory protein SP1714 is a repressor of its own expression and that of two putative ABC transporters, are involved in the defense of *S. pneumoniae* against different AMPs and enable the identification of common features of the molecular defense mechanisms against various antimicrobial substances in this organism. This will eventually lead to the selection and/or design of more suitable antimicrobial agents and the development of more effective preventive measures.

**MATERIALS AND METHODS**

**Bacteria and growth conditions.** The strains used in this study are listed in Table 1 and were stored in 10% glycerol at −80°C. *Streptococcus pneumoniae* strains were grown at 37°C in standing Todd-Hewitt (Oxoid) broth supplemented with 0.5% yeast extract (THY) and/or on M17 agar (69) containing 0.25% chloramphenicol (2 µg/ml) and 4 µg/ml tetracycline (2.5 µg/ml for *S. pneumoniae*), trimethoprim (18 µg/ml for *S. pneumoniae*), and ampicillin (100 µg/ml for *E. coli*). Nisin (Sigma) was used for induction of gene expression at a concentration of 5 ng/ml. Aliquots of *S. pneumoniae* D39 cultures were prepared as follows. Overnight cultures of D39 grown in THY were diluted 1:100 in the same medium. Subsequently, the bacteria were grown at 37°C with shaking. Where appropriate, media were supplemented with the following antibiotics (final concentrations shown in parentheses): erythromycin (2 µg/ml for *S. pneumoniae* and 4 µg/ml for *L. lactis*), tetracycline (2.5 µg/ml for *S. pneumoniae*), trimethoprim (18 µg/ml for *S. pneumoniae*), and ampicillin (100 µg/ml for *E. coli*). Nisin (Sigma) was used for induction of gene expression at a concentration of 5 ng/ml.

**Antimicrobial agents.** Stock solutions of antimicrobial peptides/agents were stored in aliquots at −20°C. The solutions of bacitracin (Sigma), Hoechst 33342 [2’-(4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2,5-(4-ethyl-1-piperazinyl)-2,5-bi-H-bezimidazol-2 yl], Molecular Probes, Inc.), gramicidin (Sigma), lincomycin (Sigma), vancomycin (Sigma), daunomycin (Sigma), and ethidium bromide (Sigma) were prepared in MilliQ water. The stock solution of nisin (Sigma) was prepared in 0.05% acetic acid and that of LL-37 (Innovagen) in 0.01% acetic acid with 0.01% bovine

**TABLE 1. Strains and plasmids used in this study**

| Strain or plasmid | Descriptiona | References or source |
|-------------------|--------------|---------------------|
| *S. pneumoniae* strains |
| D39              | Serotype 2 strain, cps2 | 1, 39; source was the group of P. W. Hermans |
| D39misRK         | D39 ΔgagA::nisRK; Tmp'   | 29 |
| Δ385-387         | D39 ΔSP0385-0387; Spt'   | This work |
| Δ785-787         | D39 ΔSP0785-0787; Ery'   | This work |
| Δ912-913         | D39 ΔSP0912-0913; Ery'   | This work |
| Δ1714-1715       | D39 ΔSP1714-1715; Ery'   | This work |
| Δ1715            | D39 ΔSP1715; Ery'       | This work |
| Δblp strain      | D39 ΔSPD0473-0476; Ery' | This work |
| OV912            | D99nisRK/pNZ912; Cmr     | This work |
| OV1715           | D99nisRK/pNZ1715; Cmr    | This work |
| CO912            | OV1715 Δ1714-1715        | This work |
| CO1715           | OV1715 Δ1714-1715        | This work |
| DM39             | Δ385-387 Δ912-913         | This work |
| DM19             | Δ912-913 Δ1714-1715       | This work |
| PR385            | D39 ΔgagA::spr0385-lacZ; Tet' | This work |
| PR785            | D39 ΔgagA::spr0785-lacZ; Tet' | This work |
| PR912            | D39 ΔgagA::spr0912-lacZ; Tet' | This work |
| PR1714           | D39 ΔgagA::spr1714-lacZ; Tet' | This work |
| PR785Δ1714       | PR785/Δ1714-1715         | This work |
| PR912Δ1714       | PR912/Δ1714-1715         | This work |
| PR1714Δ1715      | PR1714/Δ1714-1715        | This work |
| E. coli EC1000   | MC1000 derivative carrying a single copy of the pWV01 repA gene in ggbB; Km' | 40 |
| L. lactis NZ9000 | MG1363 ΔspepN::nisRK    | 35 |
| Plasmids          |                        |         |
| pPP2             | Promoterless lacZ, for replacement of bgaA (spr0565) with promoter-lacZ | fusions, derivative of pPP1; Amp' Tet' |
| pNZ8048          | Nisin-inducible PniG; Cmr | 19 |
| pPA1             | pPP2 spr0835-lacZ        | This work |
| pPA2             | pPP2 spr0785-lacZ        | This work |
| pPA3             | pPP2 spr0912-lacZ        | This work |
| pPA4             | pPP2 spr1714-lacZ        | This work |
| pNZ921           | pNZ8048 carrying SP0912-0913 downstream of PniG | This work |
| pNZ1715          | pNZ8048 carrying SP1715 downstream of PniG | This work |

a Ery', erythromycin resistance; Cmr', chloramphenicol resistance; Tet', tetracycline resistance; Spt', spectinomycin resistance; Tmp', trimethoprim resistance.
serum albumin (NEB). Dilutions of each antimicrobial compound were always freshly prepared from these stocks.

**Strain construction.** Strains, plasmids, and oligonucleotide primers used in this study are listed in Tables 1 and 2. The genome sequence of *S. pneumoniae* D39 was used to design all primers (39). All the indicated PCR fragments and plasmids were introduced into *S. pneumoniae* D39 as described previously (29, 58). *S. pneumoniae* clones were selected on GM17 agar with the appropriate antibiotic(s). *L. lactis* and *E. coli* were transformed by electroporation as described before (22). All constructs and deletions were verified by DNA sequencing.

**Construction of deletion strains.** The knockout of the *SP0385-SP0387* (fragment length, 585 nt) was amplified with the primer pair Psp912-913-fv/Psp912-913-rev, and the putative promoter of the *SP1714-1715* (fragment length, 237 nt) was amplified with the primer pair Psp1714-1715-for-3/Psp1714-1715-rev-2, and were fused to NcoI/XbaI sites in pNZ8048, respectively, and were fused to NcoI/XbaI sites in pNZ8048, giving rise to the plasmids pNZ912, pNZ1714, pNZ1715, and pNZ912. These plasmids were transformed into the strains D39 to generate the PR385, PR785, PR912, and PR1714 strains. In addition, the introduction of plasmids PA2, PA3, and PA4 into a Δ1714 mutant resulted in the PR785/Δ1714, PR912Δ1714, and PR1714Δ1714 strains, respectively.

**Construction of overexpression plasmids.** For overexpression of *SP0912-0913* and *SP1715* with the nisin-inducible system (13, 35), these genes were amplified with the primer pairs Ovrsps912-913-fv/Ovrsps912-913-rev and Ovrsps1715-fv/Ovrsps1715-rev, respectively, and were fused to NcoI/XbaI sites in pNZ8048, yielding pNZ912 and pNZ1715. These plasmids were transformed into *S. pneumoniae* D39, generating the OV912 and OV1715 strains. For the complementation assay, pNZ912 was transformed into the Δ912 strain, yielding strain CO912, and pNZ1715 was transformed into strains Δ1714-1715 and Δ1715, yielding strains CO1714 and CO1715, respectively.

**DNA microarrays and transcriptional profiling.** DNA microarrays were produced and analyzed as described before (36, 78). Experimental design. One-milliliter aliquots of *S. pneumoniae* D39 (OD<sub>600</sub> of ~0.25) were used to inoculate 100 ml of THY medium and were grown at 37°C.

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**TABLE 2. Oligonucleotide primers used in this study**

| Oligonucleotide primer | Nucleotide sequence (5’ to 3’)* | Restriction site |
|------------------------|---------------------------------|-----------------|
| KN-sp912-913-for-1     | GGAAGCCAGCCACAGGCTGTA          | Neot |
| KN-sp912-913-rev-2     | GAGATCTAATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | NcoI |
| KN-sp912-913-for-3     | AGTTATCGGCAATATCGTGATCGGCAATATCGCATCCTTCTGTCG | XbaI |
| KN-sp785-787-for-1     | CTAGCTAGTCAATATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | Neot |
| KN-sp785-787-rev-2     | GAGATCTAATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | NcoI |
| KN-sp785-787-for-3     | CTAGCTAGTCAATATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | XbaI |
| KN-sp385-387-for-1     | CTAGCTAGTCAATATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | Neot |
| KN-sp385-387-rev-2     | GAGATCTAATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | NcoI |
| KN-sp385-387-for-3     | CTAGCTAGTCAATATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | XbaI |
| KN-sp1714-1715-for-1   | CTAGCTAGTCAATATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | Neot |
| KN-sp1714-1715-rev-2   | GAGATCTAATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | NcoI |
| KN-sp1714-1715-for-3   | CTAGCTAGTCAATATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | XbaI |
| KN-sp1714-1715-rev-4   | CTAGCTAGTCAATATCGATGCCTGGTCAATGAGAATCGCATCCTTCTGTCG | Neot |
| KN-sp785-787-for-1     | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | XbaI |
| KN-sp785-787-rev-2     | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | Neot |
| KN-sp785-787-for-3     | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | XbaI |
| KN-sp385-387-for-1     | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | Neot |
| KN-sp385-387-rev-2     | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | NcoI |
| KN-sp385-387-for-3     | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | XbaI |
| KN-sp1714-1715-for-1   | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | Neot |
| KN-sp1714-1715-rev-2   | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | NcoI |
| KN-sp1714-1715-for-3   | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | XbaI |
| KN-sp1714-1715-rev-4   | GGAAGACTGTGGTTACCATCAGAAGAATCGCATCCTTCTGTCG | Neot |

* Restriction enzyme sites are underlined.
until early logarithmic phase (OD$_{600}$ of ~0.25). Subsequently, cultures were split in two and exposed to 0.7 µg/ml bacitracin, 0.1 µg/ml nisin, or 4.5 µg/ml LL-37 (end concentrations) for 15 (early response) and 30 (late response) min. These concentrations of AMPs were chosen based on the results of growth experiments performed with all three AMPs and gave a 10% reduction of the maximal OD compared to that with no AMP. In this manner, the bacteria were stressed with the AMPS but not killed to a great extent, because this would negatively influence the quality of the RNA for the transcriptome experiments. For each AMP, three replicates were performed, and as a control, bacteria without any AMP were used.

RNA isolation, cDNA preparation, and hybridization. RNA was isolated from 50 ml of three independent cultures for each condition. After centrifugation, pellets were frozen in liquid nitrogen and stored at −80°C. Subsequently, pellets were resuspended in 500 µl of 10 mM Tris-Cl and 1 mM EDTA (pH 8.0), after which 50 µl of 10% sodium dodecyl sulfo-sodium taurocholate (1% w/v) was added. Next, the pellets were collected and RNA was isolated from 15 µg of total RNA and the Cy3/Cy5-dCTP labeling of cDNA was performed with a CyScribe postlabeling kit (Amersham Biosciences). Hybridization was carried out at 45°C for 16 h in Ambion slide hybridization buffer (Ambion Europe) on supermerge glass slides (Array-it; SMMBC). The slides contained replicates of amplicons of 2,087 open reading frames (ORFs) of S. pneumoniae. The slides were scanned using a Gen-eTae LSIV confocal laser scanner (Genomics Solutions).

Data analysis. ArrayPro 4.5 (Media Cybernetics, Inc., Silver Spring, MD) was used to analyze the data. For the processing and normalization of the data, the MicroPrep software was used as described previously (78, 79). Genes with a Bayes ratio (Bayes value) with a differential expression greater than or equal to 1.2 or lower than or equal to 0.8 were considered significantly differentially expressed.

β-Galactosidase assays. S. pneumoniae strains were incubated at 37°C in THY and grown to early logarithmic phase (OD$_{600}$ of ~0.25). Subsequently, D39 derivatives were incubated for 15 (data not shown), 30, and 90 min with or without 0.7 µg/ml bacitracin, 0.1 µg/ml nisin, or 4.5 µg/ml LL-37 (the same end concentrations of these AMPs were used for transcriptome analyses). Next, the pellets were collected and β-galactosidase assays were performed as described previously by Israelsen et al. (26), with the following modifications. Two milliliters of the cell cultures were centrifuged; pellets were resuspended in 250 µl Z buffer (60 mM Na$_2$HPO$_4$, 10 mM KH$_2$PO$_4$, 5 mM KCl, MgSO$_4$, 7H$_2$O) and 15 µl (final concentration, 0.06 mg/ml) cetyltrimethylammonium bromide and incubated for 5 min at 30°C. The assay was started by addition of 50 µl of 4 mg/ml ONPG (O-nitrophenyl β-d-galactopyranoside; Sigma Chemical) and 100 µl of 30% glutaraldehyde (pH 7.0). The enzymatic reaction was stopped by addition of 175 µl of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0), after which 20 µl of the mixture was transferred to a 96-well plate and read at 420 nm.

Determination of MICs. Determination of the MICs of the various compounds for S. pneumoniae D39 and the mutants was performed in 96-well microtiter plates. Incubation took place in a microplate reader (GENios; Tecan). Aliquots of strains OV912, OV1715, CO912, CO1715, and CO1716 were made using THY broth with an induction concentration of nisin (5 ng/ml) for the nisin-inducible expression of the genes of interest. For the MIC assays, the aliquots were thawed, spun down, and resuspended in fresh THY broth. The medium of strains OV912, OV1715, CO912, CO1715, and CO1716 was again supplemented with the induction concentration of nisin. Exponentially growing strains at an OD$_{600}$ of ~0.2 were added into the wells of microtiter plates at a total volume of 200 µl/well with increasing concentrations of the antimicrobial substance being tested. The microtiter plates were incubated at 37°C for overnight growth, and the OD$_{600}$ was measured every 30 min. The MICs were determined when the reference strain carrying overexpression vectors, these strains were also examined in the MIC assay without nisin induction, but no change in the susceptibility was observed compared to that with nisin (data not shown). All the susceptibility assays were performed at least in triplicate.

Microarray data accession number. The DNA microarray data were submitted to the GEO database and are available under accession number GSE16491.

RESULTS

Genome-wide identification of S. pneumoniae genes responding to bacitracin, nisin, or LL-37 challenge. Nisin, bacitracin, and LL-37 differ in structure and mode of action, but their targets, subunits of the bacterial cell envelope, are thought to be similar. To investigate whether there is a general stress response of S. pneumoniae to different AMPS, transcriptome analyses of strain D39 exposed for 15 and 30 min to sublethal amounts of either bacitracin, nisin, or LL-37 were performed.

Exposure to all three AMPS resulted in significantly changed (Bayes P value of ≤0.0001 and fold change of ≤0.8 or ≥1.2) transcript levels of genes involved in various processes, such as regulation, transport, fatty acid biosynthesis, virulence, bacteriocin production, metabolic processes, protein fate, and phosphotransferase systems, and many genes encoding hypothetical proteins. LL-37 seemed to have the most profound influence on the transcriptome of S. pneumoniae D39, as the expression of ~10% of the genome changed upon exposure. A complete overview of significantly up- and downregulated genes are in Table S1 in the supplemental material. The response to each individual AMP had a number of genes in common at both time points (see Table S2, section A, in the supplemental material), and several genes were differentially regulated upon challenge with more than one AMP at both 15 and 30 min (see Table S2, section B). Subsequently, we investigated how many significantly down- and upregulated genes were identified as common in each stress response to bacitracin, nisin, and LL-37 after two time points (Fig. 1; also see Table S3). The data revealed that treatment with nisin and LL-37 for 15 min resulted in only a few (11) downregulated genes in common (Fig. 1; also see Table S1, sections C and E, and Table S3, section A). Prolonging the time of exposure to these two AMPS to 30 min did not yield any genes in common (Fig. 1A; also see Table S1, sections D and F). Interestingly, there were no downregulated genes identified when strain D39 was exposed for 15 min to bacitracin. After 30 min of treatment with this AMP, 66 genes were downregulated (see Table S1, section B), of which were also downregulated by exposure to LL-37 for 30 min (Fig. 1A; also see Table S1, sections B and F, and Table S3, section A). Treatment with all three AMPS induced the expression of several common genes, the number of which increased with longer exposure (Fig. 1B; also see Table S3).

Although bacitracin, nisin, and LL-37 are distinct antimicrobial compounds, the S. pneumoniae transcriptome response to them revealed certain analogous features. Since we were interested in genes that might be involved in the resistance mechanisms of D39 to two or all three AMPS, which are expected to be upregulated, we focused on the most interesting and prominently induced genes, which are described below.

Genes induced in the response to all three AMPS. Comparison of the transcriptome profiles of S. pneumoniae D39 in response to bacitracin, nisin, and LL-37 revealed that gene SP0641, encoding the pneumococcal surface serine protease PrtA (6); gene SP2062, a member of the VicRK regulon (50, 52) encoding a putative transcriptional regulator of the MarR (multiple antibiotic resistance regulators) family; and genes SP0419 and SP0422, involved in fatty acid biosynthesis (41), were all moderately (SP0641, 1.3- to ~3-fold; SP2062, 1.5- to
~2-fold; \( SP0419 \), ~1.8-fold to 2-fold; and \( SP0422 \), 1.4 to ~2.2-fold) upregulated upon exposure to each AMP at either 15 or 30 min (Fig. 2; also see Table S3 in the supplemental material). Gene \( SP0913 \), encoding a permease protein, was induced moderately (2-fold) upon LL-37 treatment and up to 13-fold upon treatment with nisin and bacitracin. \( SP0912 \), an ATP-binding protein, was upregulated 9-fold upon nisin and bacitracin exposure, and it probably forms an ABC transporter with \( SP0913 \) (Fig. 2 and Table 3). \( SP0912-0913 \) were chosen for further study. The \( SP0915 \) gene, encoding a putative membrane protein, was induced moderately (1.5-fold) upon LL-37 treatment. \( SP0914 \) gene encodes a protein with a LysM (lysin motif) domain, so it probably transcribes from the same promoter as the \( TCS03 \) operon (27). The transcript level of the adjacent \( SP0385-0387 \) gene product changed similarly to that of \( TCS03 \). The \( SP0385 \) membrane protein, with unknown function, shares 27% sequence identity to \( LiaF \) (\( YvqE \)), a membrane protein of the \( liaRS \) gene cluster. Analysis of the genomic sequence of strain D39 revealed that \( SP0385 \) is probably transcribed from the same promoter as the \( TCS03 \) genes. To investigate whether the \( SP0385-0387 \) genes play a role in \( S. pneumoniae \) resistance to AMPs, we chose them for further study.

The expression of the \( SP0785-0787 \) genes increased more than 2-fold upon bacitracin stress and more than 4-fold upon LL-37 stress (Fig. 2 and Table 3). Analysis of the D39 genomic sequence indicated that the \( SP0785-0787 \) genes might be transcribed from the same promoter, which is in accordance with the transcriptome data. \( SP0785 \) encodes a protein annotated in the NCBI database as a membrane fusion protein (MFP) subunit of an efflux transporter. The \( SP0786-0787 \) genes are annotated as encoding an ABC transporter, with \( SP0786 \) encoding an ATP-binding protein and \( SP0787 \) a permease protein with three transmembrane domains. Interestingly, the \( SP0787 \) protein showed 34% amino acid sequence identity to BacI, involved in secretion of bacteriocin 21, and 32% amino acid sequence identity to MacB, involved in resistance to macrolides (31, 71). Therefore, we decided to investigate the function of \( SP0785-0787 \) further.

The \( SP1714-1715 \) genes, presumably in an operon, were upregulated more than 2-fold in response to bacitracin and even 13-fold in response to LL-37 (Table 3) and were chosen for further study. The \( SP1714 \) gene encodes a putative transcriptional regulator of, most likely, the \( GntR \) (gluconate regulator) family of regulators, while \( SP1715 \) encodes a putative ABC transporter.

**Genes induced upon challenge with bacitracin and nisin.** Among the genes that were upregulated upon both bacitracin and nisin exposure were the \( SP0912 \) gene, described above, and the \( SP2063 \) gene (Fig. 2; also see Table S1, sections B and D, and Table S3 in the supplemental material). \( SP2063 \), a member of the \( VicRK \) regulon (50, 52), was upregulated 7-fold upon bacitracin stress and almost 2-fold upon nisin stress. This gene encodes a protein with a LysM (lysin motif) domain, so it is probably cell wall attached, but otherwise the function is unknown (9) (Fig. 2).

**Upregulated genes in common for the nisin and LL-37 response.** Treatment with nisin or LL-37 positively stimulated the expression of several identical genes. Among them was the \( SP2174 \) gene, encoding \( DltD \) (Fig. 2; also see Table S1, sections C and E, and Table S4, section A, in the supplemental material). Interestingly, all four genes of the \( dlt \) operon, \( dlt-ABCD \) (\( SP2173-2176 \)), showed induction upon LL-37 exposure (see Table S1, section E), but only one gene of this operon, \( dltD \), was upregulated upon nisin exposure (Fig. 2; also see Table S1, sections C and E). The \( dlt \) operon encodes proteins mediating n-alanylation of the teichoic acids, which improves resistance to neutrophil traps in TIGR4 (80). Furthermore, the

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**FIG. 1.** Venn diagrams indicating the numbers of genes downregulated (A) and upregulated (B) in the 15- and 30-min stress response of D39 to bacitracin, nisin, and LL-37. Numbers quantify the genes with significantly altered expression (Bayes \( P \) value of ≤0.0001; expression ratio greater than 1.2 or lower than 0.8) that were either shared or exclusive to each D39 response. Lists of genes in common indicated in this figure can be found in Table S3, sections A, B, and C, in the supplemental material.
**dlt** operon confers resistance to nisin and gallidermin in strains Rx and D39 and, in *S. aureus*, to defensins, protegrins, and other cationic AMPs (33, 55). Thus, the upregulation of **dlt** genes upon LL-37 stress and of **dltD** upon nisin stress is in accordance with previous data and indicates that this operon also plays a role in the resistance of *S. pneumoniae* D39 to LL-37.

**Differences in the D39 transcriptome response to bacitracin, nisin, and LL-37.** The **glnRA** (*SP0501-0502*), **htrA** (*SP2063*), **SP2240**, and **blp** (*SP0525-0529*, **SP0533**, and **SP0545-0547**) genes had opposite expression levels upon challenge with different AMPs. Surprisingly, the **glnRA** genes were upregulated upon LL-37 stress, whereas they were downregulated upon challenge with nisin (Fig. 2; also see Table S1, sections C, D, E, and F, in the supplemental material). The **glnR** gene encodes the repressor of the genes encoding the glutamine synthesis and uptake complex, **glnA** and **glnPQ** (30). Although the genes involved in glutamine metabolism are well studied within pathogens (20, 30, 67, 68, 75), it is not clear why **glnRA** are oppositely expressed upon nisin and LL-37 exposure.

Similarly, the expression of **htrA** and its adjacent gene **SP2240** was antagonistic in the D39 stress response to bacitracin and LL-37 (Fig. 2; also see Table S1, sections B, E, and F, in the supplemental material). These genes were downregulated 2-fold upon bacitracin treatment and upregulated more than 3-fold upon LL-37 exposure. HtrA (high-temperature requirement A), a major virulence factor of *S. pneumoniae*, is a serine protease that plays a significant role in resistance to high temperatures and oxidative stress and is involved in transformation efficiency (12, 24). One of the pneumococcal TCSs, CiaRH (*SP0798-0799*), positively controls the expression of **htrA** and **SP2240** (23, 46, 62). Since **ciaRH** was upregulated upon challenge with LL-37 and not with bacitracin, the induction of **htrA** and **SP2240** expression in response to LL-37 was most likely mediated by CiaRH. The expression of the **SP0107** gene, also a member of the VicRK regulon (50, 52), which encodes a protein with a LysM (9) domain and unknown function, increased more than 2-fold upon bacitracin exposure and was reduced approximately 2-fold upon LL-37 exposure (Fig. 2).

One feature completely distinguished the response to LL-37 from that to bacitracin and to nisin; genes of the **blp** (bacteriocin-like peptide; **pnc**) locus were induced only upon LL-37 stress (Fig. 2 and Table 3; also see Table S1, sections E and F, in the supplemental material). The **blp** genes encode proteins for Blp bacteriocin production, regulation, transport, and immunity (11, 12, 14, 42). Since the putative **blp** immunity genes, **SP0545-0547**, were strongly induced only upon LL-37 stress (Table 3), we speculated that, in strain D39, they might be involved in a specific resistance mechanism against this AMP, and therefore, **blp** genes involved in putative bacteriocin production and immunity were selected for further study.

**FIG. 2.** General comparison of differentially and antagonistically expressed genes/gene products of strain D39 involved in regulation, virulence, and resistance mechanisms upon bacitracin, nisin, and LL-37 stress for 15 and/or 30 min. The direction of the arrow indicates up- or downregulation, and the thickness of the arrow indicates the strength of the differential expression.
Changes mediated by bacitracin, nisin, and LL-37 on the expression of SP0385-0387, SP0785-0787, SP0912-0913, and SP1714-1715. In order to confirm the differential patterns of expression upon bacitracin, nisin, and LL-37 challenge, lacZ-promoter fusions of the promoters of the genes selected for further study were made. The same experimental procedure as applied for the transcriptome analysis was used for AMP exposure, with one modification. Exposure of the D39 derivatives to the AMPs for 30 and 90 min resulted in higher exposure, with one modification. Exposure of the D39 derivatives applied for the transcriptome analysis was used for AMP exposure and approximately 2-fold with the other two AMPs), exposure to all AMPs tested (more than 3-fold upon bacitracin stimulation, but part of the amplicon sequence of SP0533 is identical to that of SPD0046).

The expression of the SP0385-0387 promoter increased upon exposure to all AMPs tested (more than 3-fold upon bacitracin exposure and approximately 2-fold with the other two AMPs), which is in contrast to the transcriptome profiling, where these ORFs were induced only upon bacitracin and LL-37 exposure (Table 4). The activity of the SP0785-0787 promoter increased slightly, approximately 2-fold, upon bacitracin and nisin stimulation, but there was no effect of LL-37 exposure (Table 4), which differs from the results observed in the transcriptome analysis. Induction of $P_{SP0912-0913}$ activity upon LL-37 stress was not observed, but in response to bacitracin and nisin, its dependent increase, indicating that this is not a general effect of the AMPs on the β-galactosidase assay (data not shown).

The expression of the SP0385-0387 promoter increased upon exposure to all AMPs tested (more than 3-fold upon bacitracin exposure and approximately 2-fold with the other two AMPs), which is in contrast to the transcriptome profiling, where these ORFs were induced only upon bacitracin and LL-37 exposure (Table 4). The activity of the SP0785-0787 promoter increased slightly, approximately 2-fold, upon bacitracin and nisin stimulation, but there was no effect of LL-37 exposure (Table 4), which differs from the results observed in the transcriptome analysis. Induction of $P_{SP0912-0913}$ activity upon LL-37 stress was not observed, but in response to bacitracin and nisin, its dependent increase, indicating that this is not a general effect of the AMPs on the β-galactosidase assay (data not shown).

**TABLE 3. Differential expression of genes selected for further analysis upon *S. pneumoniae* treatment for different times with bacitracin, nisin, and LL-37**

| TIGR4 locus tag | D39 locus tag | Putative/predicted function | Gene |
|----------------|---------------|-----------------------------|------|
| SP0385         | SPD0350       | Membrane protein            |      |
| SP0386         | SPD0351       | Sensor histidine kinase      |      |
| SP0387         | SPD0352       | DNA-binding response regulator |      |
| SP0525         | SPD0467       | Regulator protein            |      |
| SP0526         | SPD0468       | Response regulator           |      |
| SP0527         | SPD0469       | Histidine kinase             |      |
| SP0528         | SPD0470       | Peptide pheromone            |      |
| SP0529         | SPD0471       | ABC transporter, permease protein |      |
| SP0530         | SPD0472       | ABC transporter, ATP-binding protein |      |
| SP0533         | SPD0473       | Bacteriocin                  |      |
| SP0545         | SPD0474       | CAAX protease                |      |
| SP0546         | SPD0475       | CAAX protease                |      |
| SP0785         | SPD0686       | RND efflux-like protein      |      |
| SP0786         | SPD0687       | ABC transporter, ATP-binding protein |      |
| SP0787         | SPD0688       | ABC transporter, permease protein |      |
| SP0912         | SPD0804       | ABC transporter, ATP-binding protein |      |
| SP0913         | SPD0805       | ABC transporter, permease protein |      |
| SP1714         | SPD1524       | GntR transcriptional regulator |      |
| SP1715         | SPD1525-1526  | ABC transporter, ATP-binding protein |      |

|          | Bacitracin | Nisin | LL-37 |
|----------|-----------|-------|-------|
| Fold induction upon exposure for indicated time (min) to: | 15 | 30 | 15 | 30 | 15 | 30 |

* Values are the averages of the results of five independent experiments, and the standard deviations are indicated in parentheses. ND, not determined.

b The activities of the promoters of the SP0785-0787 and SP1714-1715 genes were also studied in a ΔSP1714-1715 strain. In all cases, the bacteria were grown in THY without AMPs or with either 0.7 μg/ml bacitracin, 0.1 μg/ml nisin, or 4.5 μg/ml LL-37.

**TABLE 4. β-Galactosidase activities of the promoters of the SP0385-0387, SP0785-0787, SP0912-0913, and SP1714-1715 genes in the wild-type D39 strain, transcriptionally fused to lacZ**

| Strain | Genes regulated by promoter | Activity (Miller units) of promoter with indicated treatment for indicated time (min) |
|--------|----------------------------|----------------------------------------------------------------------------------|
|        |                            | Without AMP | Bacitracin | Nisin | LL-37 |
|        |                            | 30 | 90 | 30 | 90 | 30 | 90 | 30 | 90 | 30 | 90 |
| D39    | SP0385-0387                | 32 | (6) | 45 | (16) | 194 | (31) | 259 | (46) | 96 | (3) | 82 | (14) | 99 | (36) | 85 | (11) |
|        | SP0785-0787                | 24 | (3) | 26 | (3) | 45 | (6) | 59 | (8) | 46 | (8) | 36 | (11) | 37 | (9) | 37 | (7) |
|        | SP0912-0913                | 4 | (1) | 4 | (2) | 23 | (2) | 52 | (12) | 59 | (10) | 64 | (20) | 4 | (0.5) | 3 | (0.1) |
|        | SP1714-1715                | 25 | (4) | 59 | (5) | 90 | (16) | 114 | (24) | 68 | (7) | 60 | (24) | 146 | (40) | 267 | (21) |

a The activities of the promoters of the SP0785-0787 and SP1714-1715 genes were also studied in a ΔSP1714-1715 strain. In all cases, the bacteria were grown in THY without AMPs or with either 0.7 μg/ml bacitracin, 0.1 μg/ml nisin, or 4.5 μg/ml LL-37.

b Values are the averages of the results of five independent experiments, and the standard deviations are indicated in parentheses. ND, not determined.
activity was more than 12- and 15-fold higher (Table 4), respectively, which corresponds to the transcriptome data. After 30 min of induction, the expression of SP1714-1715 was enhanced 3-fold upon bacitracin exposure and 6-fold upon LL-37 exposure (Table 4), which is in agreement with the transcriptome data. However, the expression of this promoter also increased approximately 2-fold after 30 min of treatment with vancomycin, daunomycin, or ethidium bromide (Table 5). None of the strains overexpressing these promoters (OV912) increased the expression from these promoters in a wild-type background (CO1715, SP0785-0787), which demonstrated that the GntR-like regulator (SP1714) in the observed increased susceptibility of the mutant to bacitracin, Hoechst 33342 and sensitivity to LL-37. Introduction of either the SP0385-0387 or the SP1714-1715 mutation into the ΔSP0912-0913 background (DM39 and DM19, respectively) did not result in increased sensitivity to bacitracin, nisin, Hoechst 33342, or LL-37 compared to that of the single mutants, indicating that these proteins are functioning in the same pathway. Additionally, overexpression of SP0912-0913 genes (OV912) increased the resistance of D39 to bacitracin more than 3-fold, whereas it had only a moderate effect on resistance to nisin and gramicidin and no effect on resistance to lincomycin. Overexpression of SP1715 in both mutant and wild-type backgrounds (CO1715, CO1716, and OV1715) increased the sensitivity to LL-37 7-fold compared with that of the wild type, resistance to Hoechst 33342 increased 2-fold, and minor effects were observed for bacitracin. Thus, multiple genes identified in the transcriptome analysis indeed play a role in the resistance of D39 to the tested AMPs. Furthermore, some of these genes also confer resistance to other antimicrobial compounds.

The GntR-like regulator, SP1714, is a repressor of its own expression and that of SP0785-0787. The SP1714-1715 and SP0785-0787 genes were upregulated upon treatment with bacitracin and LL-37, and mutation of these genes changed the resistance of D39 to these two AMPs. This indicated that the SP1714-1715 and SP0785-0787 genes might belong to the same regulatory pathway. Therefore, we decided to study the influence of the SP1714 regulator on the expression of several gene promoters (SP0785-0787, SP0912-0913, and SP1714-1715). The activity of P<sub>SP1714-1715</sub> in the ΔSP1714-1715 background increased about 6-fold, and this induction was independent of the stress caused by the AMPs (Table 4). Likewise, the activity of P<sub>SP0912-0913</sub> in the ΔSP1714-1715 background increased about 4-fold, which demonstrated that the GntR-like regulator repressed P<sub>SP0912-0913</sub> expression, which was again independent of AMP addition (Table 4). Unfortunately, the open reading frame of SP1714 overlaps with that of SP1715, making it difficult to delete only SP1714 without influencing SP1715 expression. Therefore, in order to avoid mutant construction difficulties and to exclude the possibility that SP1715 played a part in the observed regulatory effects, we examined the expression from these promoters in a ΔSP1715 mutant. As expected, there was no effect of SP1715 deletion on the expression of the promoters of SP0785-0787 and SP1714-1715. Likewise, there was no effect of either SP1714-1715 or SP1715 deletion on the expression of the SP0912-0913 promoter (data not shown). These data suggest that SP1714, encoding a GntR-like regulator, is a repressor of its own expression, as well as that of SP1715 and SP0785-0787.

### Table 5. MICs for *S. pneumoniae* D39 and derivatives treated with various antimicrobial substances

| MIC (µg/ml)<sup>a</sup> | strain Bacitracin | Nisin | LL-37 | Hoechst 33342 (µM) | Gramicidin | Lincomycin |
|--------------------------|------------------|-------|-------|-------------------|-----------|-----------|
| D39                      | 4                | 0.8   | 1     | 2.2               | 0.5       | 0.5       |
| Δ385-387                 | 1.5              | 0.8   | 1     | 2.2               | 0.5       | 0.5       |
| Δ1715                    | 1.7              | 0.8   | 30    | 0.2               | 0.5       | 0.5       |
| ΔSP0785-0787             | 1.7              | 0.8   | 30    | 0.5               | 2.2       | 0.5       |
| CO1715<sup>c</sup>       | 4                | ND    | 1     | ND                | ND        | ND        |
| CO1716<sup>c</sup>       | 5                | ND    | 2     | ND                | ND        | ND        |
| OV1715<sup>c</sup>       | 5                | ND    | 2     | ND                | ND        | ND        |
| Δblp strain              | 4                | 0.8   | 3     | 2.2               | 0.5       | 0.5       |
| Δ912-913                 | 0.7              | 0.2   | 14    | 1                 | 0.03      |           |
| CO912<sup>e</sup>        | 4                | 0.6   | ND    | 2                 | 4         |           |
| OV912<sup>e</sup>        | 15               | 1     | ND    | 2.5               | 0.5       |           |
| DM39<sup>e</sup>         | 0.7              | 0.2   | 9     | ND                | 1.0       | 0.5       |
| DM19<sup>e</sup>         | 0.7              | 0.4   | 26    | ND                | 2         | 2         |

<sup>a</sup> Values are the averages of the results of at least three independent experiments. MICs are given in micrograms per milliliter unless stated otherwise. Bold font indicates a difference of more than approximately 2-fold compared to the MIC for the wild type. ND, not determined.

<sup>b</sup> Strain overexpresses SP1715 in the Δ1715 mutant.

<sup>c</sup> Strain overexpresses SP1715 in the Δ1714-1715 mutant.

<sup>d</sup> Strain overexpresses SP0912-0913 in the Δ912-913 mutant.

<sup>e</sup> Double mutant of Δ385-387 with Δ912-913.

<sup>f</sup> Double mutant of Δ912-913 with Δ1714-1715.

<sup>g</sup> Strain overexpresses SP0785-0787 in wild-type *S. pneumoniae* D39.
The objective of this study was to investigate whether the stress response of *S. pneumoniae* D39 to bacitracin, nisin, and LL-37 would reveal common features. A second objective was to determine whether any genes identified have a direct role in conferring resistance to these and various other antimicrobial compounds. Bacitracin, nisin, and LL-37 differ in structure and mode of action, but their targets, subunits of the bacterial cell envelope, are similar. Comparison of the transcriptome response to each compound revealed that they had a low number of significantly differentially expressed genes in common (Fig. 1 and 2). The response of strain D39 to LL-37 was rather broad compared to that to bacitracin or nisin. This extensive reaction to LL-37 suggests a more general response of D39 to human peptides than to bacterial compounds, i.e., bacitracin and nisin (Fig. 1). Analysis of the differentially expressed genes after either 15 min or 30 min of exposure to the tested AMPs showed little overlap in downregulated genes in comparison to that in induced genes (Fig. 1). Comparison of the early response (15 min) to the late one (30 min) for each AMP showed that there was little overlap of commonly up- or downregulated genes (see Fig. S1 and Table S2, section A, in the supplemental material). However, among these commonly induced genes, we identified several, *SP0912-0913, SP0785-0787, and SP1714-1715*, that were involved in the resistance of D39 to the AMPs tested, as shown by susceptibility assays. Thus, the transcriptome response of D39 to the AMPs changes with time but genes determining resistance are induced in both the early (15 min) and the late (30 min) responses. Interestingly, the reaction of D39 to LL-37 and bacitracin had more genes in common than the reaction to LL-37 and nisin or to bacitracin and nisin (Fig. 1), which might suggest a more similar general stress response to bacitracin and LL-37.

The genes *SP0385-0387, SP0912-0913, SP0785-0787, SP1714-1715*, and *blp* had large changes in expression upon challenge with one or more AMPs; therefore, they were characterized in more detail since they could be alternative candidates for resistance inhibition by specific drugs. Notably, transcription of homologues of some of the genes identified in this study, e.g., *SP0386-0387, SP0912-0913, SP0785-0787, and SP1714-1715*, has also been found to be affected in response to various antimicrobial compounds, including bacitracin, nisin, or LL-37, in several other Gram-positive bacteria, i.e., *L. lactis, B. subtilis*, and *B. licheniformis* (34, 45, 57).

We showed that the *SP0912-0913* genes, encoding a putative ABC transporter, were induced upon exposure to all three AMPs tested (Fig. 2 and Table 3) and that the mutant was more sensitive to bacitracin and nisin and, additionally, to lincomycin and gramicidin (Table 5). The finding that *SP0912-0913* is involved in resistance to lincomycin, nisin, and bacitracin is in accordance with previous data for the *SP0912-0913* homologue from *B. subtilis*, BceAB (formerly YtsCD), which was induced upon bacitracin and LL-37 challenge and which conferred resistance to bacitracin in this bacterium (5, 57). The other homologues of *SP0912-0913*, MbrAB from *S. mutans* and YsaBC from *L. lactis*, modulated bacitracin and nisin resistance, respectively (34, 74). Although it was shown that *SP0912-0913* genes were induced in *S. pneumoniae* TIGR4 and Tupelo strains upon vancomycin challenge (18), we have not seen increased sensitivity of the *SP0912-0913* mutant to this antibiotic (data not shown). Thus, the *SP0912-0913* transporter does not appear to be directly involved in resistance to vancomycin. The finding that *SP0912-0913* is involved in resistance to antimicrobial compounds acting on the cell envelope, i.e., nisin and bacitracin, and antimicrobial compounds involved in protein synthesis inhibition, i.e., lincomycin (10), strongly suggests that the ABC transporter might be of the MDR type. Recently, Becker et al. showed that this ABC transporter is indeed involved in resistance of *S. pneumoniae* R6 to bacitracin (3).

Both the TCS03 gene and the upstream gene *SP0385*, which probably forms an operon with TCS03, were induced upon bacitracin and LL-37 challenge (Fig. 2 and Table 3). The exact function of TCS03 in *S. pneumoniae* is not yet known, but it has been shown that the expression of the *SP0385-0387* genes was positively affected upon vancomycin stress but repressed during invasive disease in cerebrospinal fluid (CSF) (18, 54). TCS03 shares significant amino acid sequence similarity to TCS11 from *S. mutans*, to CesSR from *L. lactis*, to VraRS from *S. aureus*, and to LiaRS (YvyEC) from *B. subtilis*. It has been shown that these homologous TCSs are induced upon challenge with various AMPs, although they did not confer significant resistance to the antimicrobial agents tested (44, 45, 57, 77). This study also showed that SP0385-0387 did not confer significant resistance to the compounds tested, except for bacitracin (Table 5), which corresponds to the phenotype of TCS03 homologues in *L. lactis* CesSR, *S. aureus* VraRS, and *B. subtilis* LiaRS (37, 44, 45, 47). Therefore, it has been proposed that these TCSs are the sensors of cell envelope-mediated stresses, but their exact role in the response to AMPs remains unclear (27). Interestingly, three genes that belong to the VicRK regulon (*SP0107, SP2062, and SP2030*) were induced by AMP in our study. The VicRK TCS and its homologues in other Gram-positive bacteria regulate, among others, genes involved in murein biosynthesis and are essential (81); in *S. pneumoniae* this is due to its regulation of PesB (51). In *S. mutans*, it was shown that the VicRK homologue is under the positive control of the LiaRS system (72). Thus, it might well be that to withstand exposure to AMPs and the subsequent stress on the cell wall, the VicRK regulon is also necessary.

The *SP0785-0787* genes, encoding a putative ABC transporter, were induced in response to both bacitracin and LL-37 (Fig. 2 and Table 3), and the *SP0785-0787*-deficient strain was significantly more sensitive to LL-37 and lincomycin and moderately sensitive to gramicidin (Table 4). The *SP0785-0787* genes were upregulated upon vancomycin stress (18), but the susceptibility assay did not show increased sensitivity of the *SP0785-0787* mutant to this antibiotic. Interestingly, Marrer et al. (43) demonstrated that the *SP0785-0787* genes were induced upon bacitracin, chloramphenicol, and fusidic acid exposure but repressed by actinomycin and ciprofloxacin challenges (43). These data indicate that *SP0785-0787* might be involved in *S. pneumoniae* resistance to even more antimicrobial compounds than were tested here, which could imply that the *SP0785-0787* proteins display some characteristics of MDR and are of direct importance for the global defense mechanism against antimicrobial compounds in *S. pneumoniae*.

The *SP1714* and *SP1715* genes, encoding a GntR-like regulator and a putative ABC transporter, respectively, were con-
siderably upregulated upon challenge with LL-37 and bacitracin (Fig. 2 and Table 3). Strains deficient in SP1715 and both SP1714 and SP1715 were more sensitive to Hoechst 33342 and bacitracin. Surprisingly, the SP1715 and SP1714-1715 mutants were more resistant to LL-37 than the wild type, whereas complementation and overexpression of SP1715 increased the sensitivity of strain D39 to LL-37. These data indicate that, on the one hand, SP1715 is involved in D39’s sensitivity to LL-37 and, on the other, in D39’s resistance to bacitracin and Hoechst 33342. Furthermore, we show that SP1714 is a negative regulator of its own gene and, most likely, also of SP1715 that determines sensitivity to LL-37 and seems to be in the same operon and of SP0785-0787, which protect against LL-37. Since SP1714 was upregulated upon challenge with LL-37 and bacitracin, we speculate that the stress caused by these antimicrobial compounds induces an unknown factor which subsequently interacts with SP1714. This interaction might cause release of SP1714 from a dedicated promoter site and, consequently, derepression of genes regulated negatively by SP1714, i.e., SP1714-1715 and SP0785-0787.

Most of the described GntR-like regulators are repressors of various bacterial metabolic pathways, such as gluconate, histidine, and arabinose biosynthesis (61). Recently, Truong-Bolduc and Hooper identified a new GntR-like regulator, NorG, that regulates the expression of quinolone and β-lactam multidrug efflux pumps (73). In previous studies, the expression profile of SP1714-1715 increased after induction with vancomycin (18), but treatment with penicillin had an opposite effect (60). In addition, these genes were downregulated in the CSF fraction during a transcriptome study of *S. pneumoniae* during invasive disease (54). These data could imply that the expression of SP1714-1715 depends on external stimuli and that the GntR-like protein, SP1714, might regulate the response to a wide variety of toxic components, most likely via an additional regulatory mechanism. The exact function of the GntR-like regulator, SP1714, remains to be determined and is the subject of ongoing studies.

Interestingly, the *blp* genes were induced only upon stimulation with LL-37 (Fig. 2 and Table 3). Notably, from the eight TCS mutants tested for growth efficiency in a respiratory tract infection (RTI) model, only a BlpR mutant was attenuated, indicating that it is an essential TCS under these conditions (70). The reason why BlpRH was essential for pneumococcal survival within the RTI remained unclear. Our transcriptome data showed that the presence of LL-37 induced the entire *blp* locus, especially the putative *blp* immunity genes. Previously, it has been demonstrated that the chemically synthesized peptide pheromone BlpC first induces the two-component system, BlpRH, which subsequently leads to upregulation of the complete *blp* gene cluster (14). Since LL-37 and BlpC are short linear cationic peptides, we hypothesize that like BlpC, LL-37 could interact with BlpH and, consequently, through BlpR, activate the entire *blp* locus. We also speculate that the *blp* immunity proteins could confer D39 resistance to LL-37, which is strongly supported by the finding that the *blp*-deficient strain was sensitive to LL-37. This would explain why BlpRH is essential in the RTI, where many AMPs, such as LL-37, are present. In order to confirm our hypothesis, we will continue to evaluate whether LL-37 can induce the expression of BlpRH and if the expression of the entire *blp* locus will be enhanced in consequence. In addition, we will examine whether the *blp* mutant is more sensitive to other human AMPs.

To conclude, the transcriptional response of *S. pneumoniae* D39 to three distinct AMPs, bacitracin, nisin, and LL-37, was diverse and complex and revealed that only a few genes were differentially expressed in response to all three. Most importantly, mutants of some of these genes in D39, i.e., *SP0912-0913, SP0785-0787*, and *SP1714-1715*, exhibited cross-sensitivity/resistance to several antimicrobial substances, including some that were not used in the initial challenge experiments, which, to our knowledge, has not been shown before. Additionally, we showed that the *blp* locus is involved in determining the resistance of D39 to a human AMP, LL-37. Therefore, some of these genes might be interesting candidates for inhibition by specific blocking reagents, which would result in novel medicines for the prevention and treatment of pneumococcal diseases.

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