

The Mevalonate Pathway of *Staphylococcus aureus*^{▽†}

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Isoprenoids are a class of ubiquitous organic molecules synthesized from the five-carbon starter unit isopentenyl pyrophosphate (IPP). Comprising more than 30,000 known natural products, isoprenoids serve various important biological functions in many organisms. In bacteria, undecaprenyl pyrophosphate is absolutely required for the formation of cell wall peptidoglycan and other cell surface structures, while ubiquinones and menaquinones, both containing an essential prenyl moiety, are key electron carriers in respiratory energy generation. There is scant knowledge on the nature and regulation of bacterial isoprenoid pathways. In order to explore the cellular responses to perturbations in the mevalonate pathway, responsible for producing the isoprenoid precursor IPP in many gram-positive bacteria and eukaryotes, we constructed three strains of *Staphylococcus aureus* in which each of the mevalonate pathway genes is regulated by an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter. We used DNA microarrays to profile the transcriptional effects of downregulating the components of the mevalonate pathway in *S. aureus* and demonstrate that decreased expression of the mevalonate pathway leads to widespread downregulation of primary metabolism genes, an upregulation in virulence factors and cell wall biosynthetic determinants, and surprisingly little compensatory expression in other isoprenoid biosynthetic genes. We subsequently correlate these transcriptional changes with downstream metabolic consequences.

Isoprenoids are a class of organic molecules synthesized from the five-carbon starter unit isopentenyl pyrophosphate (IPP). Found ubiquitously in nature, more than 30,000 known isoprenoid natural products have been isolated from eubacteria, archaeobacteria, and eukaryotes serving a myriad of biological functions (14, 28). Isoprenoids are involved in membrane architecture as hopanoids and sterols, especially cholesterol, partake in electron transport as ubiquinones (coenzyme Q), menaquinones (vitamin K), and heme A, regulate protein biosynthesis as prenylated tRNAs, participate in cell wall peptidoglycan biosynthesis as the carrier lipid undecaprenyl pyrophosphate, are posttranslationally appended to proteins as dehydrodolichyl and farnesyl pyrophosphates, comprise pigments such as chlorophyll and carotenoids, and act as intercellular messengers in the form of fungal mating factors, plant cytokines, insect hormones, and steroid hormones (7, 9, 14, 28).

The biosynthetic route to the common isoprenoid precursor IPP was first discovered in eukaryotes in the 1960s (12). In this pathway, three molecules of acetyl coenzyme A (acetyl-CoA) condense to the intermediate 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which is subsequently reduced to the intermediate mevalonate, for which the pathway is named. Mevalonate is then doubly phosphorylated, decarboxylated, and dehydrated to form IPP (4, 7, 28). More recently, an independent pathway originating from condensation of glyceraldehyde-3-phosphate and pyruvate to form the intermediate 1-deoxyxylulose-5-phosphate (DXP), for which the pathway is named, was

found to be utilized in many gram-negative bacteria, some gram-positive bacteria, algae, and some higher plants (14, 20, 23). Most research in understanding regulation of IPP biosynthesis has been directed toward the mevalonate pathway in eukaryotes, and consequently the isoprenoid biosynthetic pathway in mammalian cells has served as an important area for therapeutic intervention of various diseases. HMG-CoA reductase, the enzyme that catalyzes the rate-limiting step in human IPP biosynthesis (7), is the target of the popular statin class of cholesterol-lowering drugs (6). Farnesyl pyrophosphate synthase, which performs two consecutive condensations between IPP and its isomer dimethylallyl pyrophosphate, is targeted by the bisphosphonate class of drugs licensed to treat cancer and bone disease (21). In addition, inhibitors of farnesyltransferase, an enzyme required for the posttranslational farnesylation of proteins which properly localizes them to membranes, are in various stages of clinical development (2, 3).

In contrast, there is a paucity of knowledge concerning the nature and regulation of bacterial isoprenoids, as illustrated by the discovery of the distinct DXP pathway only recently. Due to its novelty, a great deal of focus has been placed on this new pathway which functions more predominantly in gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (13, 14). However, in the gram-positive pathogens *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Enterococcus faecalis* the only route to isoprenoid production is the mevalonate pathway (14, 19, 28). Given its essentiality in cell wall biosynthesis, respiratory energy generation, and involvement in oxidative stress protection, there are presumably complex regulatory mechanisms interconnected to isoprenoid biosynthesis in bacteria. In order to explore the cellular responses to perturbations in the mevalonate pathway, we constructed several strains of *S. aureus* in which each of the mevalonate path-

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TABLE 1. Primers and probes for qRT-PCRs

Gene	5' Primer	3' Primer	Probe (5'FAM-3'TAMRA)
<i>mvaS</i>	AATACGCAAAACGTCAAGGTA AGTC	TCTAATGCCTTTTTACCCATTT TTGT	TGACTTCGCATCTCTATGCTTC CATGTTCC
<i>mvaA</i>	TTGCTGCCGTTGGTTTAGC	GACCTTGTGAATACCTTCTGA CACA	ACTTTGCAGCATGTCGCGCGC
<i>mvaK1</i>	CAAATTGCACATGGTAAACC AAGT	TTCAACGTTTCAGCATGACCTT	ACGCAAACGATTGTATCAGGC AAACCA
<i>mvaD</i>	TGCATGCCACGAATCTAGGA	GCGCCATGACATCATAACTTTC	AACACCGCCGTTACATATCTT GTGCA
<i>mvaK2</i>	TGGATTGCACATCGAACCAT	AAACGTTTCACTTCGCTAACA AAGT	CTGGCTCACC GGCGTCATCACC
SAOUHSC_02243	GCGTCATCATTATCATGTGCAA	TCCTTCTTATTTTGGTCTTGAG AGTCTTT	CAGCAACGACTCAAGCAAATT CAGCTCA
<i>narG</i>	CTTGGTGGTGAATGCTTAGT TTTT	CATCTGTTTGCTCTCCCAAA	TATGCAGATTTACCACCTGCCT CTCCACA
16S rRNA	GTGAATACGTTCCCGGCTCTT	CGGCTTCGGGTGTTACAAAC	ACACCGCCCGTCACACCACGA

way genes is under the control of an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter. We subsequently used DNA microarrays to profile the transcriptional effects of downregulating the components of the mevalonate pathway in *S. aureus*.

MATERIALS AND METHODS

Generation of mutants. Mevalonate pathway mutants in *mvaS* (HMG-CoA synthase), *mvaA* (HMG-CoA reductase), and *mvaK1* (mevalonate kinase) were generated by a promoter exchange method utilizing the pMUTIN vector (27). For each gene subjected to promoter exchange, the 5' portion of the corresponding gene was cloned into the integration plasmid pMUTIN, which is nonreplicative in *S. aureus* but can be manipulated and propagated in *E. coli*. For *mvaS* the gene and 25 bases of the 5' untranslated region (UTR) were cloned using the primers 5'-CCT AAGCTTAAATTTGAAAGGATGTAAACCT-3' and 5'-TAAGAATTCCTTAGCTTCTTCAATTTCAAAGCAGC-3'; for *mvaA* the gene and 12 bases of the 5'UTR were cloned using the primers 5'-CCTAAGCTT AGGAGTACGTCCATG CAAAATTTAGAT-3' and 5'-TAAGAATTCCTTATTTTTCAGTATCGTCAACGCCA-3'; and for *mvaK1* the gene and 15 bases of the 5'UTR were cloned using the primers 5'-TTTAAGCTTAAAGGTGATATTGACATGACAAGA-3' and 5'-TAAGAATTCCTTAGCCGTCTAAACTTAACGTTTTCAAC-3' (the restriction sites are underlined). DNA fragments were amplified from *S. aureus* RN4220 genomic DNA and cloned into the HindIII and EcoRI sites in pMutin, which are located downstream of the Pspac promoter. Upon sequence confirmation (Agencourt Bioscience Corp., Beverly, MA), the resulting plasmids were transformed (22) into *S. aureus* RN4220 cells, and the plasmid integrants based on homologous recombination were selected on agar plates containing 5 μ g of erythromycin/ml and 100 μ M IPTG. The successful integration of each promoter exchange vector was confirmed by PCR analysis. The generated *S. aureus* mevalonate pathway mutants for *mvaS*, *mvaA*, and *mvaK1* were designated RN4220spacmvaS, RN4220spacmvaA, and RN4220spacmvaK, respectively.

Bacterial growth. *S. aureus* cells pelleted from an overnight culture grown in NZYM containing 5 μ g of erythromycin/ml and 1 mM IPTG were washed three times, each in 500 μ l of fresh NZYM. The washed cells were then diluted 200 or 400 times with the same medium, and 10 μ l of the resulting cell suspension was transferred to each well of a 96-well microtiter plate containing 90 μ l of NZYM and a desirable amount of IPTG or mevalonate. The microtiter plate was then incubated at 37°C. The growth of the bacterial cells was monitored by recording the optical density at 600 nm (OD₆₀₀) at various time points using a SpectraMAX plate reader (Molecular Devices, Union City, CA). The growth defect in the mevalonate mutants was highly reproducible and recoverable with IPTG induction through several experiments.

RNA isolation. Overnight cultures of *S. aureus* were diluted 5,000-fold into fresh NZYM $\pm$ 1 mM IPTG. Cultures were grown for 6 to 8 h with vigorous shaking at 37°C and an equivalent of 15 ml of culture at an OD₆₀₀ of 1.0 were harvested by centrifugation. Cells were resuspended in 4 ml of H₂O and 8 ml of RNeasy Protect bacteria reagent (Qiagen, Valencia, CA), reacted for at least 30 min, pelleted by centrifugation, and then frozen at -80°C until further use. RNA was extracted from cell pellets by using a Purescript RNA isolation kit (Gentra

Systems, Minneapolis, MN) with an initial 0.3- μ g/ μ l treatment with lysostaphin (Sigma, St. Louis, MO) to achieve cell lysis. A total of 100 μ g of total RNA was treated with 7 U of RNase-free DNase I (Qiagen) and then further purified by using an RNeasy Plus minikit (Qiagen). RNA quality was interrogated by reacting with formaldehyde loading dye running on a 1% agarose denaturing gel with morpholinepropanesulfonic acid gel running buffer from a NorthernMax kit (Applied Biosystems, Austin, TX). Final RNA samples were quantified by using a nanodrop spectrophotometer (Nanodrop, Wilmington, DE).

Microarray analysis. Gene expression profiling was performed using Affymetric GeneChips designed to interrogate over 3,300 *S. aureus* open reading frames and both forward and reverse orientation of over 4,800 intergenic regions. Then, 15 μ g of RNA was reverse transcribed, and the cDNA was fragmented, labeled, and hybridized to the GeneChip *S. aureus* genome array using the standard prokaryotic GeneChip protocol supplied by Affymetric. GeneChip data were obtained by scanning with an Affymetric autoloading scanner, and data were normalized and compared by using GeneSpring 7.2 analysis software (Agilent Technologies, Santa Clara, CA). All experiments were done in triplicate using independent RNA samples.

qRT-PCR analysis. Primers and probes (5'-FAM, 3'-TAMRA) for quantitative reverse transcription-PCR (qRT-PCR) (Table 1) were designed by using Primer Express v.2.0 software (Applied Biosystems, Foster City, CA) and were synthesized by Integrated DNA Technologies (Coralville, IA). The levels of *mvaS*, *mvaA*, *mvaK1*, *mvaD*, *mvaK2*, *narG*, and SAOUHSC_02243 transcripts were monitored by qRT-PCR analysis using a Superscript III Platinum One-Step qRT-PCR System (Invitrogen, Carlsbad, CA) on an Applied Biosystems Prism 7900HT sequence detection system. Relative quantitation was done by the comparative cycle threshold method using serial dilutions of a plasmid containing *mvaS* cloned into pET15b. All experimental samples were normalized to 16S rRNA internal control. Cycle threshold values were calculated by using Applied Biosystems sequence detection systems software v.2.3. Each sample contained 666 ng of total RNA, except when quantifying 16S rRNA, for which 1.33 ng was used. The thermocycling conditions were as follows: 50°C for 20 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Preliminary experiments were carried out to optimize primer and probe concentrations to give the best efficiencies. RNA samples were tested by PCR with Phusion DNA polymerase to ensure no contamination with genomic DNA. All experiments were done in triplicate.

Metabolic pattern profiling. Overnight cultures of *S. aureus* were diluted 5,000-fold in fresh NZYM plus 1 mM IPTG or 2,000-fold in fresh NZYM with no IPTG. Cultures were grown for 6 h with vigorous shaking at 37°C, and an equivalent of 20 ml of culture at an OD₆₀₀ of 0.6 was harvested by centrifugation, washed with distilled water, and resuspended in 19 ml of GN/GP inoculating fluid (Biolog, Hayward, CA). Then, 150 μ l of diluted cells was dispensed into each well of a GP2 microplate (Biolog, Hayward, CA) and grown overnight at 37°C. Metabolic profiling experiments were performed in triplicate for all strains tested, yielding the same results.

Autolysis assay. Overnight cultures of *S. aureus* were diluted 5,000-fold in 4 ml of fresh 1.25 $\times$ NZYM plus 1 mM IPTG or 2,000-fold in 4 ml of fresh 1.25 $\times$ NZYM with no IPTG. Cultures were grown at 37°C for 1.5 h, and then 1 ml of 5 M NaCl was added. Cultures continued to grow at 37°C until reaching an OD₆₀₀ of 1.0, at which point the cells were spun down, washed with 5 ml of

ice-cold distilled water, and resuspended in enough 50 mM Tris (pH 8.0)–0.1% Triton X-100 to give an OD₆₀₀ of 0.5 when reading 100 µl in a microtiter plate on a SpectraMAX plate reader. Lysis of bacterial cells was monitored by recording the OD₆₀₀ at various time points. All samples were tested in quadruplicate.

All supporting microarray data were submitted to the Gene Expression Omnibus under accession number GSE13424.

RESULTS

Construction of regulated mevalonate pathway *S. aureus* strains. A promoter exchange scheme was utilized to generate *S. aureus* mutants in which each of the mevalonate pathway genes was placed under the control of the IPTG-inducible Pspac promoter. In each instance the 5' portion of the corresponding gene was cloned into the integration plasmid pMUTIN, propagated in *E. coli*, and then transformed into *S. aureus* RN4220. Since the plasmid is nonreplicative in *S. aureus*, proper integration based on homologous replication was selected for by acquisition of erythromycin resistance. Three strains were produced: RN4220spacmvaS, which regulates HMG-CoA synthase (*mvaS*); RN4220spacmvaA, which regulates HMG-CoA reductase (*mvaA*); and RN4220spacmvaK, which regulates mevalonate kinase (*mvaK1*). Since *mvaK1* is the first gene in a tricistronic transcript containing *mvaK1-mvaD-mvaK2*, its regulation should simultaneously affect the expression of mevalonate diphosphate decarboxylase and phosphomevalonate kinase, respectively. Transcriptional profiling and qRT-PCR data later confirmed the regulated expression of these genes in their respective strains (see below).

Since each of the genes involved in mevalonate biosynthesis is essential to bacterial viability, it is expected that growth of the constructed strains should be dependent on IPTG induction. Indeed, in the absence of IPTG, the growth of all regulated strains was severely reduced (Fig. 1). This growth defect could be rescued by induction of the mevalonate pathway genes (Fig. 1), an effect that scaled with the concentration of IPTG (data not shown). Significant increases in growth could be observed starting at 16 µM IPTG but were optimal at 1 mM IPTG. It has been reported that mevalonate can enter *S. aureus* cells and bypass the requirement of its endogenous synthesis (29). As depicted in Fig. 1, chemical complementation with mevalonate was able to overcome the growth defect resulting from a lack of MvaS or MvaA. Once again, the rescue in growth scaled with the concentration of mevalonate (data not shown), reaching full restoration with 200 µM mevalonate. In contrast, the hindered growth due to underexpression of the *mvaK1-mvaD-mvaK2* operon could not be rescued by the addition of mevalonate. These results are consistent with the fact that MvaS and MvaA act upstream of mevalonate biosynthesis, whereas MvaK1, MvaD, and MvaK2 catalyze transformations downstream of mevalonate in isoprenoid biosynthesis.

Transcriptional profiling of regulated mevalonate pathway *S. aureus* strains. With mevalonate regulation in *S. aureus* established, we next turned to profiling the transcriptional changes that occur under conditions of overexpression and underexpression of the mevalonate pathway genes. Total RNA was extracted from mid-exponential-phase *S. aureus* cultures grown in the presence or absence of 1 mM IPTG and analyzed using the Affymetrix *S. aureus* GeneChip. Interestingly, as a whole the transcriptome of RN4220spacmvaS, RN4220spacmvaA, and RN4220spacmvaK induced with 1 mM IPTG did not differ significantly from WT *S.*

aureus grown with 1 mM IPTG. Although IPTG induction does not increase *mvaS* expression in RN4220spacmvaS beyond levels observed in WT cells, *mvaA* expression did increase 2.1-fold in RN4220spacmvaA, and *mvaK1*, *mvaD*, and *mvaK2* expression increased 6.8, 2.6, and 2.1-fold, respectively, in RN4220spacmvaK. Under these conditions of overexpression, the only significant changes (at least 2-fold) between RN4220spacmvaA and WT were a 2.2-fold decrease in *coa* (SAOUHSC_00192) expression and a 2.5-fold increase in the expression of a hypothetical protein (SAOUHSC_00561). Similarly when comparing induced RN4220spacmvaK and WT, the only significant changes were 2.0- and 2.2-fold increases in the nitrate reductase subunits *narJ* (SAOUHSC_02679) and *narH* (SAOUHSC_02680), respectively. Therefore, for all further analyses we chose to compare transcriptional changes between uninduced and induced cultures, with WT cells present as a control.

Overall, the transcriptome of *S. aureus* responded similarly to downregulation of any of the mevalonate pathway genes, with a total of 224 upregulated genes and 180 downregulated genes common to all three mutants. These alterations in transcription represent a threshold of at least 2-fold and have a *P* value of ≤0.05 as determined by one-way analysis of variance. This large number of genes with altered expression is demonstrative of the complexity of feedback mechanisms connected to isoprenoid biosynthesis present in *S. aureus*.

Changes in isoprenoid biosynthesis. Since we have perturbed the initial steps in the essential mevalonate pathway, we first wanted to examine the transcriptional changes incurred in isoprenoid biosynthesis. Table 2 lists the changes in transcription for genes involved in synthesis of the common isoprenoid precursor IPP, the carrier lipid for cell wall biogenesis undecaprenyl pyrophosphate, the electron transporters menaquinone and heme A, prenylated tRNAs, and the carotenoid staphyloxanthin. It is interesting that in the essential UPP pathway there was not only a 2-fold upregulation of undecaprenyl pyrophosphate synthase but also a significant increase in the expression of *bacA*, which is involved in undecaprenyl recycling (5). Conversely, there was a twofold downregulation in the operon coding for biosynthesis of the nonessential carotenoid staphyloxanthin, and although there was only a minor decrease in the isoprenoid utilizing farnesyltransferase *ctaB*, required for heme O biosynthesis, there was a twofold decrease in the subsequent heme O-utilizing enzyme *ctaA*, which completes heme A formation. In comparison, genes for menaquinone biosynthesis, which in *S. aureus* is coded for by the canonical *men* pathway and not the newly described *mqn* pathway present in *Streptomyces coelicolor* A3(2) (8), showed little to no change in their transcript levels upon downregulation of any of the mevalonate pathway genes. These results in Table 2 suggest that *S. aureus* is able to detect a limiting supply of isoprenoid precursors and redirect biosynthetic pathways to maximally ensure growth and survival.

When we examined the genes responsible for synthesis of the common isoprenoid precursor IPP, the same genes that are being regulated by the Pspac promoter in the generated mutant strains, it was evident that the lack of IPTG does in fact decrease transcripts of the corresponding genes to levels of ca. 10% found in induced *S. aureus* (Table 2). Furthermore, as expected, regulation of *mvaK1* simultaneously regulates *mvaD* and *mvaK2*. It appears that regulation of IPP biosynthesis is

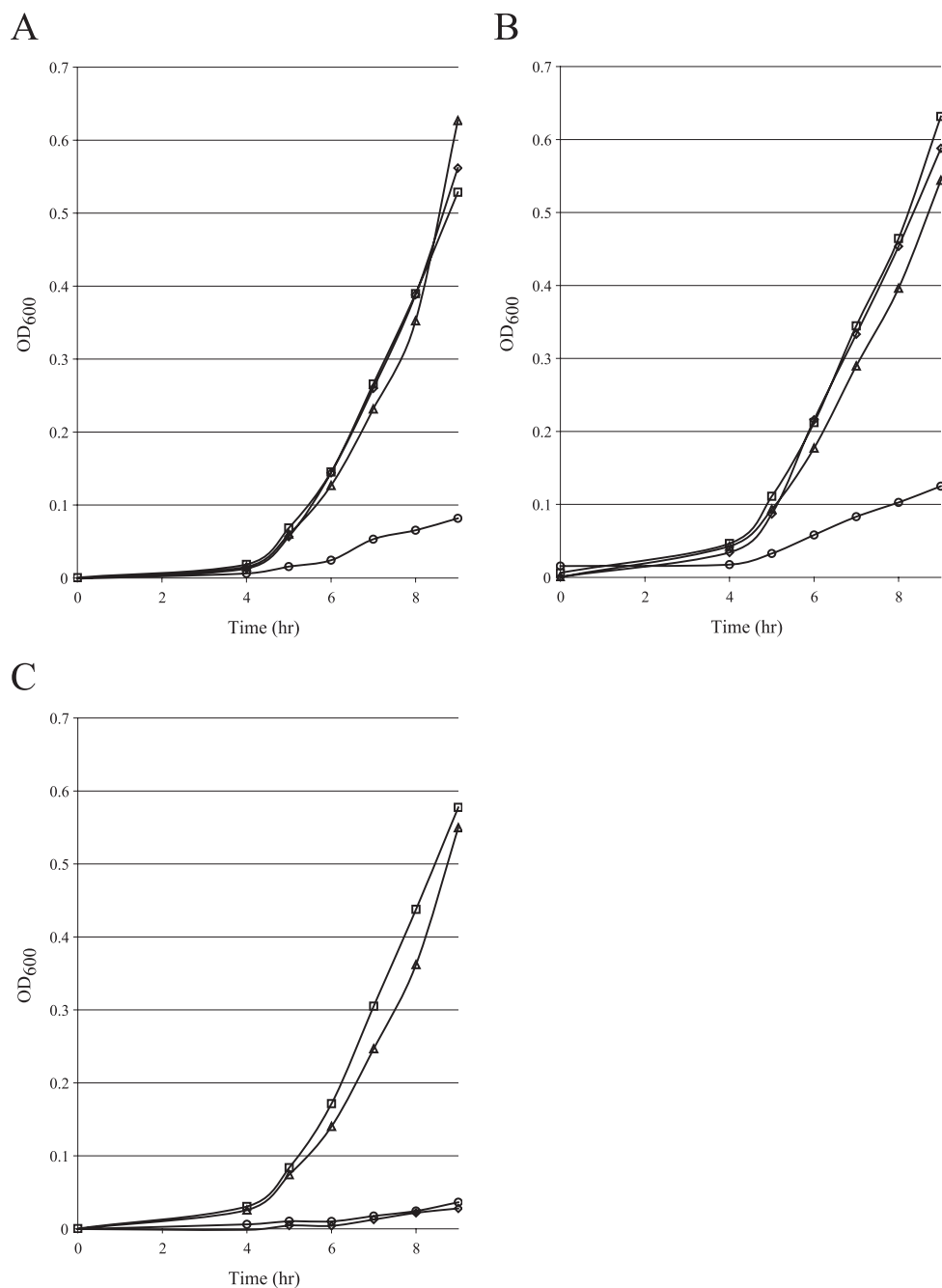


FIG. 1. Growth of mevalonate pathway-regulated mutants RN4220spacmvaS (A), RN4220spacmvaA (B), and RN4220spacmvaK (C). Circles represent growth in no IPTG and no mevalonate, diamonds represent growth in no IPTG and 1 mM mevalonate, triangles represent growth in 100 μ M IPTG and no mevalonate, and squares represent growth in 100 μ M IPTG and 1 mM mevalonate. Curves are the average of two experiments.

anterograde, meaning that genes later in the pathway are up-regulated in response to earlier deficiencies. Hence, *mvaS* is nonresponsive to deficiencies in genes catalyzing later steps, *mvaA* is slightly upregulated in response to lower levels of *mvaS* but not *mvaK1*, and the *mvaK1-mvaD-mvaK2* operon is upregulated in response to deficiencies in either *mvaS* or *mvaA*.

In order to verify these results for the mevalonate pathway genes, as well as the microarray results as a whole, we per-

formed qRT-PCR for all of the strains using probes to *mvaS*, *mvaA*, *mvaK1*, *mvaD*, and *mvaK2*, as well as one random gene, SAOUHSC_02243, highly overexpressed in the absence of IPTG and one random gene, *narG* (SAOUHSC_02681), highly underexpressed in the absence of IPTG. All samples were normalized to 16S rRNA quantities, which were also calculated by qRT-PCR. Table 3 illustrates that the overexpression control SAOUHSC_02243 was indeed highly upregulated by at least fourfold in the uninduced strains and the underexpression

TABLE 2. Transcriptional changes in isoprenoid-utilizing pathways

Function and RN4220 ORF (SAOUHSC no.)	Gene	Description	Fold change ^a			
			S-/S+	A-/A+	K-/K+	WT-/WT+
IPP synthesis						
02860	<i>mvaS</i>	HMG-CoA synthase	0.11	1.01	1.18	0.88
02859	<i>mvaA</i>	HMG-CoA reductase	1.41	0.15	1.11	0.90
00577	<i>mvaK1</i>	Mevalonate kinase	1.67	2.40	0.39	0.96
00578	<i>mvaD</i>	Diphosphomevalonate decarboxylase	1.69	1.96	0.08	0.83
00579	<i>mvaK2</i>	Phosphomevalonate kinase	1.48	1.62	0.11	0.79
UPP synthesis						
02623	<i>fni</i>	Isopentenyl diphosphate isomerase	0.76	0.72	0.62	0.85
01618	<i>ispA</i>	Farnesyl pyrophosphate synthase	1.27	1.25	0.86	0.95
01237	<i>upps</i>	Undecaprenyl diphosphate synthase	1.86	2.26	1.85	0.97
00691	<i>bacA</i>	Undecaprenyl pyrophosphate phosphatase	1.86	2.70	2.01	0.82
Menaquinone synthesis						
00982	<i>menF</i>	Isochorismate synthase	0.91	0.75	0.76	0.90
00983	<i>menD</i>	2-Succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase	0.94	0.60	0.60	0.87
01915	<i>menC</i>	O-Succinylbenzoic acid synthase	0.93	0.82	0.69	1.12
01916	<i>menE</i>	O-Succinylbenzoic acid-CoA ligase	1.42	1.34	1.19	1.02
00985	<i>menB</i>	Napthoate synthase	0.91	0.76	0.80	0.98
00984		Alpha/beta hydrolase	0.90	0.63	0.55	0.90
00980	<i>menA</i>	1,4-Dihydroxy-2-naphthodate octaprenyltransferase	1.10	1.00	0.80	0.80
01487	<i>menG</i>	Methyltransferase	1.09	1.23	0.96	1.00
01486	<i>hepB</i>	Heptaprenyl diphosphate syntase	1.10	1.19	1.10	0.93
Heme A synthesis						
01066	<i>ctaB</i>	Protoheme IX farnesyltransferase	0.78	0.77	0.70	0.98
01065	<i>ctaA</i>	Heme A synthase	0.41	0.47	0.42	0.91
Prenylated tRNA synthesis						
01280	<i>miaA</i>	tRNA Δ ² -isopentenylpyrophosphate transferase	1.33	1.82	1.32	0.97
Staphyloxanthin synthesis						
02879	<i>crtM</i>	Squalene synthase	0.65	0.58	0.46	0.96
02877	<i>crtN</i>	Squalene desaturase	0.78	0.68	0.54	0.99
02880	<i>crtQ</i>	Glycosyltransferase	0.46	0.37	0.32	0.97
02881	<i>crtP</i>	Phytoene dehydrogenase	0.47	0.38	0.36	0.97
02882	<i>crtO</i>	Acyltransferase	0.61	0.44	0.41	1.23

^a S refers to RN4220spacmvaS, A refers to RN4220spacmvaA, K refers to RN4220spacK, and WT refers to RN4220. "+" refers to cells grown in the presence of 1 mM IPTG; "-" refers to cells grown in the absence of IPTG. The greatest changes in isoprenoid-utilizing pathways occur in the *mvaK* operon, *upps*, *bacA*, and the *crt* operon.

control *narG* was highly downregulated by at least fivefold in the uninduced strains. Confirming our microarray results further, qRT-PCR revealed that *mvaS* and *mvaA* showed little if any response to perturbations in the other genes of the meva-

lonate pathway, whereas the *mvaK1-mvaD-mvaK2* operon was upregulated 1.5-fold in response to *mvaS* deficiency and 2-fold in response to *mvaA* deficiency. These results suggest that isoprenoid regulation occurs fairly late in the mevalonate pathway and acts by upregulating the three kinases responsible for converting mevalonate to IPP.

Cell wall stimulon. Since the mevalonate pathway is direct precursor to UPP synthesis, a lipid carrier essential for peptidoglycan biosynthesis, it would be expected that downregulation of the mevalonate pathway would induce transcriptional changes similar to those of cell wall targeting antibiotics. In previous transcriptomic studies, changes in gene expression were identified for several strains of *S. aureus* treated with oxacillin, D-cycloserine, bacitracin (26), or vancomycin (11, 15, 16). When these data were combined, a set of 15 upregulated and 2 downregulated genes common to all four studies was proposed to encompass a coordinately regulated core cell wall stimulon. As can be seen in Table 4, our transcriptional profiling data show that downregulation of either *mvaS*, *mvaA*, or *mvaK1* caused full activation of every gene in the cell wall

TABLE 3. qRT-PCR quantification of mevalonate pathway transcriptional changes

RN4220 ORF (SAOUHSC no.)	Gene	Fold change ^a			
		S-/S+	A-/A+	K-/K+	WT-/WT+
02860	<i>mvaS</i>	0.18†	1.46†	1.11§	0.97§
02859	<i>mvaA</i>	1.1§	0.29†	1.03†	0.98†
00577	<i>mvaK1</i>	1.44†	2.18†	0.62†	1.01†
00578	<i>mvaD</i>	1.69†	1.87†	0.23†	0.95†
00579	<i>mvaK2</i>	1.53†	1.85†	0.25†	0.91†
02243		4.63†	15.24†	5.62†	1.1§
02681	<i>narG</i>	0.08†	0.19†	0.13†	1.12§
	16S rRNA	1.00	1.00	1.00	1.00

^a S refers to RN4220spacmvaS, A refers to RN4220spacmvaA, K refers to RN4220spacK, and WT refers to RN4220. "+" refers to cells grown in the presence of 1 mM IPTG; "-" refers to cells grown in the absence of IPTG. †, $P < 0.005$; §, $P < 0.05$.

TABLE 4. Transcriptional changes in the cell wall stimulon

RN4220 ORF (SAOUHSC no.)	Gene	Description	Fold change ^a			
			S-/S+	A-/A+	K-/K+	WT-/WT+
Upregulated						
00998	<i>fnt</i>	Autolysis and methicillin resistant-related protein	2.50	3.77	2.38	0.93
01838	<i>htrA</i>	Heat shock protein, similar to serine proteinase Do	5.76	6.94	6.82	0.91
01972	<i>prsA</i>	Peptidyl-prolyl cis/trans isomerase	6.85	7.85	8.18	0.91
02012	<i>sgtB</i>	Penicillin-binding protein 1A/1B	7.33	11.14	8.50	1.04
02099	<i>vraS</i>	Two-component sensor histidine kinase	4.10	4.97	3.84	0.95
02100		Conserved hypothetical protein	4.10	5.56	4.98	1.00
02101		Conserved hypothetical protein	5.12	8.99	6.46	0.94
02112		Conserved hypothetical protein	4.05	8.67	5.20	1.17
02365	<i>murZ</i>	UDP- <i>N</i> -acetylglucosamine-1-carboxyvinyl transferase	2.17	2.82	2.13	0.95
02583		Similar to <i>lytR</i> , <i>lyt</i> divergon expression regulator	4.23	5.50	4.62	0.81
02635	<i>tcaA</i>	Glycopeptide resistance protein	6.01	9.97	7.69	0.85
02723		Glycerate kinase	1.85	2.09	1.67	0.91
02724		Conserved hypothetical protein	3.70	3.97	3.62	0.98
02811		Similar to GTP-pyrophosphokinase	4.33	8.17	5.25	1.04
02872		Conserved hypothetical protein	143.35	389.86	572.40	0.85
Downregulated						
00994	<i>atl</i>	Autolysin	0.59	0.42	0.31	0.87
00427		<i>N</i> -Acetylmuramoyl-L-alanine amidase	0.46	0.72	0.31	0.92

^a S refers to RN4220spacmvaS, A refers to RN4220spacmvaA, K refers to RN4220spacK, and WT refers to RN4220. "+" refers to cells grown in the presence of 1 mM IPTG; "-" refers to cells grown in the absence of IPTG.

stimulon. These data imply that all of the early biosynthetic steps in the mevalonate pathway have significant effects on peptidoglycan biogenesis and that *S. aureus* responds as it would to direct inhibition of cell wall biosynthesis.

Global transcriptional changes. Table S1 in the supplemental material lists all 231 genes that are collectively downregulated in response to downregulation of *mvaS*, *mvaA*, and *mvaK1*. This list is slightly expanded to include some genes that just miss the twofold difference threshold in some of the mutants but belong to pathways that are downregulated overall. From these data it is evident that there is a global response to isoprenoid inhibition that involves reducing primary metabolism. All de novo synthesis of nucleotides is severely decreased as the *pur* and *pyr* genes are downregulated by as much as 10-fold (see Table S1 in the supplemental material, p. 2 and 3). Respiration too is downregulated in several ways with genes responsible for nitrate respiration downregulated as much as 20-fold (see Table S1 in the supplemental material, p. 4) and cytochrome *c* oxidase and ubiquinol oxidase downregulated up to 3-fold (see Table S1 in the supplemental material, p. 2). Ribosomal proteins as well as heme and several amino acid biosynthetic enzymes are also significantly downregulated (see Table S1 in the supplemental material, p. 2 and 3). Interestingly, all genes involved in β -oxidation are downregulated by as much as 20-fold (see Table S1 in the supplemental material, p. 1). This pathway is responsible for the degradation of fatty acids to yield acetyl-CoA, a precursor for the mevalonate pathway. Perhaps this downregulation represents a response to the inability to utilize the product pools of acetyl-CoA for isoprenoid biosynthesis. Our preliminary data does suggest an $\sim$ 10-fold increase in the intracellular concentrations of acetyl-CoA for the mevalonate pathway downregulated mutants compared to wild-type or induced *S. aureus*.

Table S2 in the supplemental material lists all 293 genes that are collectively upregulated in response to downregulation of

mvaS, *mvaA*, and *mvaK1*. This list, too, includes some genes that narrowly elude the twofold difference threshold in some of the mutants but comprise pathways that are upregulated overall. Globally, downregulation of the mevalonate pathway appears to induce the expression of many virulence factors, including γ -hemolysin (*hlgA-C*) (see Table S2 in the supplemental material, p. 5), enterotoxin (SAOUHSC_00354) (see Table S2 in the supplemental material, p. 1), exotoxins (*set7-10*) (see Table S2 in the supplemental material, p. 1), leukotoxin (*lukD/E*) (see Table S2 in the supplemental material, p. 4), proteases (*splA-E*, *sspB*) (see Table S2 in the supplemental material, p. 4), fibronectin-binding protein (*fmb*) (see Table S2 in the supplemental material, p. 5), capsular polysaccharide biosynthesis proteins (*cap5B/D/F*) (see Table S2 in the supplemental material, p. 1), and sialic acid metabolism proteins (*nanT/A/K/R/E*) (see Table S2 in the supplemental material, p. 1). Upregulation of such a large number of virulence determinants in response to suboptimal expression of the mevalonate pathway is perhaps indicative of the bacterium taking a defensive stance given its compromised state; it may be trying to fend off an unknown source of stress. Further supporting this idea is the upregulation of several stress response proteins including antibiotic resistance associated proteins (*vraC*, *bacA*, *fnt*, *femA*, *tcaA*, and *xpaC*) (see Table S2 in the supplemental material, p. 1 to 3 and 5), DNA damage inducible protein (*dinP*) (see Table S2 in the supplemental material, p. 4), superoxide dismutases (*sodA/M*) (see Table S2 in the supplemental material, p. 1 and 3), and osmoprotectant transporters (*opuCA/BB*) (see Table S2, p. 1 in the supplemental material). Interestingly, the arginine deiminase pathway was found to be highly upregulated in the mevalonate pathway mutants (see Table S2, p. 5 in the supplemental material). This pathway converts arginine to carbamoyl phosphate, which is subsequently used to generate ATP. It has been postulated that this pathway acts as an ATP source in small colony variants lacking a functional respiratory chain (10, 24), an idea that is supported by the fact that respiration

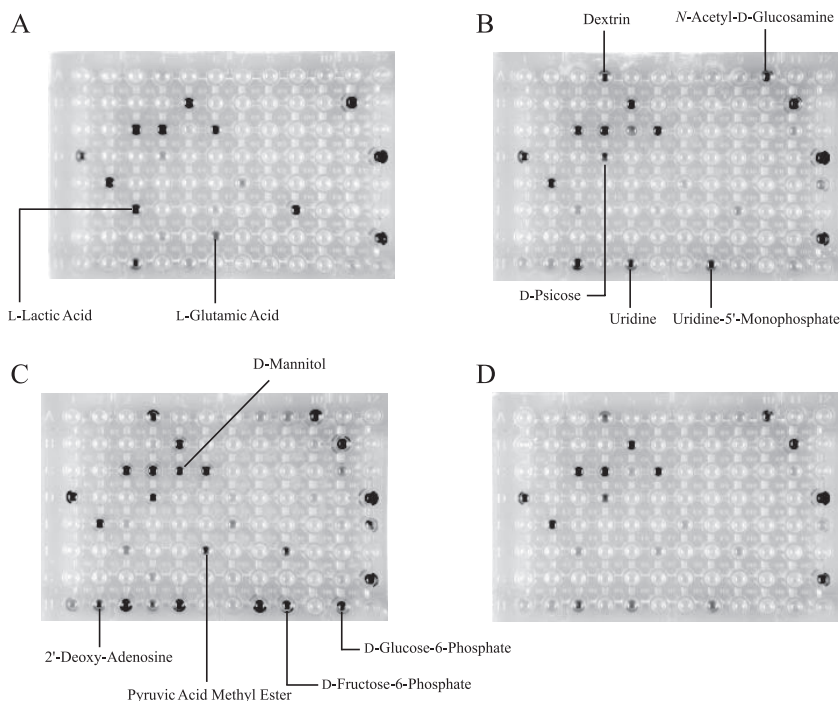


FIG. 2. Metabolic footprinting of RN4220 (A) and uninduced RN4220spacmvaS (B), RN4220spacmvaA (C), and RN4220spacmvaK (D). Carbon sources metabolized by RN4220 but not the uninduced mevalonate pathway mutants are labeled in panel A. Carbon sources metabolized by all three mevalonate pathway mutants but not RN4220 are labeled in panel B. Carbon sources metabolized only by RN4220spacmvaA are labeled in panel C.

is severely downregulated in uninduced RN4220spacmvaS, RN4220spacmvaA, and RN4220spacmvaK. Finally, further confirming that downregulation of the mevalonate pathway has a significant effect on cell wall biosynthesis, *drp35*, a stress lactonase shown to be upregulated in response to beta-lactams, bacitracin, vancomycin, and fosfomycin (17, 18), was upregulated between 6 and 10-fold in all 3 downregulated mutants (see Table S2 in the supplemental material, p. 5).

Mutant specific changes. Although the transcriptome of *S. aureus* responded comparably to downregulation of any of the mevalonate genes, there were some gene-specific changes observed. As can be seen in Table S3 in the supplemental material, 20 genes were upregulated and 5 genes were downregulated only in response to downregulation of *mvaS*, and 38 genes were upregulated and 18 genes were downregulated only in response to downregulation of *mvaA*. Interestingly, there were no transcriptional changes that occurred only in response to downregulation of the *mvaK* operon. This observation could perhaps be explained by the fact that the substrates and products for the enzymes encoded by the *mvaK1-D-K2* operon are specific to the mevalonate pathway, whereas the substrate for *mvaS*, acetyl-CoA, is also utilized by other pathways in the cell. As a result, changes in the activity of MvaS and perhaps MvaA, which utilizes the product of MvaS and could drive the reaction forward, would directly affect substrate pools of acetyl-CoA in other metabolic pathways in addition to isoprenoid biosynthesis, whereas changes in MvaK1, MvaD, and MvaK2 would only affect isoprenoid biosynthesis. Many of the genes changed specifically in response to altered transcription of either *mvaS* or

mvaA are transporters. In addition, downregulation of *mvaA* appears to cause specific changes in genes that either utilize acetyl-CoA or feed into glycolysis to produce acetyl-CoA.

Metabolic changes. The transcriptional changes incurred by *S. aureus* in response to downregulation of the mevalonate pathway genes had very distinct consequences on the metabolic capacity of the bacteria. These changes were best demonstrated by analyzing the altered ability of *S. aureus* to ferment various carbon sources (Fig. 2). Wild-type RN4220, as well as the three mevalonate mutants—RN4220spacmvaA, RN4220spacmvaS, and RN4220spacmvaK—were grown with or without IPTG induction using Biolog GP2 microplates, which measure the reduction of a tetrazolium dye in response to metabolic activity. As can be seen in Fig. 2, the wild type, as well as the fully induced mevalonate mutants (not shown), was able to ferment 13 carbon sources, including D-fructose, α -D-glucose, maltose, maltotriose, D-mannose, β -methyl-D-glucoside, sucrose, D-trehalose, L-lactic acid, pyruvic acid, L-glutamic acid, glycerol, and inosine. When the three mevalonate mutants were grown without IPTG induction, all three mutants lost the ability to ferment L-glutamic acid and were severely reduced in their ability to utilize L-lactic acid but gained the ability to ferment five new carbon sources, which included dextrin, N-acetyl-D-glucosamine, D-psicose, uridine, and uridine-5-monophosphate (Fig. 2A and B). The loss of L-glutamic acid and L-lactic acid metabolism is consistent with the transcriptional profiling data. Table 5 lists 11 genes that utilize L-glutamic acid or one of its direct by-products which are downregulated in response to suboptimal expression of *mvaS*, *mvaA*, and *mvaK*. Similarly, lactate dehydrogenase is down-

TABLE 5. Transcriptional changes in L-glutamate- and L-lactate-utilizing genes

Enzyme type and RN4220 ORF (SAOUHSC no.)	Gene	Description	Fold change ^a			
			S-/S+	A-/A+	K-/K+	WT-/WT+
Glutamate-utilizing enzymes						
00375	<i>guaA</i>	GMP synthase	0.66	0.43	0.42	0.89
01287	<i>femC</i>	Glutamine synthetase	0.52	0.39	0.45	0.97
00509	<i>gltX</i>	Glutamyl-tRNA synthetase	0.66	0.55	0.48	0.91
01014	<i>purF</i>	Amidophosphoribosyltransferase	0.26	0.14	0.14	1.01
00895	<i>gluD</i>	Glutamate dehydrogenase	0.78	0.41	0.62	0.97
01169	<i>carA</i>	Carbamoyl-phosphate synthase	0.10	0.15	0.10	1.10
01129	<i>arcC1</i>	Carbamate kinase	0.24	0.18	0.18	1.19
00008	<i>hutH</i>	Histidine ammonia-lyase	0.89	0.37	0.47	0.93
02606	<i>hutI</i>	Imidazolonepropionase	0.67	0.16	0.38	1.06
02607	<i>hutU</i>	Urocanate hydratase	0.60	0.21	0.41	1.08
02610	<i>hutG</i>	Formiminoglutamase	0.61	0.59	0.56	1.09
01776	<i>hemA</i>	Glutamyl-tRNA reductase	0.57	0.58	0.59	1.00
Lactate-utilizing enzyme						
00206	<i>ldh-1</i>	L-Lactate dehydrogenase	0.38	0.65	0.43	1.07

^a S refers to RN4220spacmvaS, A refers to RN4220spacmvaA, K refers to RN4220spack, and WT refers to RN4220. "+" refers to cells grown in the presence of 1 mM IPTG; "-" refers to cells grown in the absence of IPTG.

regulated between two- and threefold in all three mevalonate pathway downregulated mutants. The reduction in transcription levels of L-glutamic acid- and L-lactic acid-utilizing genes may correspond to their lost fermentative capacity. The acquired ability to ferment five new carbon sources, all components of peptidoglycan biosynthesis, may be a response to inhibition of cell wall biosynthesis caused by a reduction in UPP biosynthesis. Both dextrin and D-psicose can be converted into D-glucose, a precursor of N-acetyl-D-glucosamine (GlcNAc), which is a major component of the peptidoglycan. Furthermore, incorporation of GlcNAc into the cell wall requires its activation to UDP-GlcNAc, which could explain the acquired ability to ferment uridine and uridine-5-monophosphate.

Interestingly, downregulation of *mvaA* allowed *S. aureus* to metabolize five additional carbon sources not utilized by either wild-type RN4220 or the mutants downregulated in *mvaS* or *mvaK* expression (Fig. 2C). These compounds include D-mannitol, 2'-deoxyadenosine, pyruvic acid methyl ester, D-fructose-6-phosphate, and D-glucose-6-phosphate. Three of these com-

pounds are substrates for the initial transformations of glycolysis; glucose-6-phosphate is metabolized to fructose-6-phosphate by the second enzyme in glycolysis, glucose-6-phosphate isomerase; mannitol can be metabolized by phosphorylation to mannitol-1-phosphate and subsequently dehydrogenated to fructose-6-phosphate (25); and fructose-6-phosphate is the substrate for the third enzyme in glycolysis, 6-phosphofructokinase. As can be seen in Table 6, this apparent difference in fermentative capacity between the three mevalonate pathway mutants correlates well with the transcriptional profiling data. Only in response to downregulation of *mvaA* is there an increase in transcription of ~2-fold in four intermediate enzymes of glycolysis, triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and phosphoglycerate mutase, which occur directly downstream of fructose-6-phosphate biosynthesis.

In addition to altered fermentative capacity we sought to demonstrate another clear metabolic consequence of the transcriptional changes incurred by downregulation of the mevalonate pathway. Often reduced rates of cell wall biosynthesis correlate with compensatory reduced autolytic activity. The transcriptional

TABLE 6. Transcriptional changes in glycolysis

RN4220 ORF (SAOUHSC no.)	Gene	Description	Fold change ^a			
			S-/S+	A-/A+	K-/K+	WT-/WT+
01646	<i>glcK</i>	Glucokinase	0.73	0.55	0.54	0.95
00900	<i>pgi</i>	Glucose-6-phosphate isomerase	1.62	1.32	1.54	0.91
01807	<i>pfk</i>	6-Phosphofructokinase	1.13	1.45	1.12	0.96
02366	<i>fbaA</i>	Fructose-bisphosphate aldolase	0.78	0.76	0.69	1.00
00797	<i>tpi</i>	Triosephosphate isomerase	0.69	1.74	0.59	0.99
00795	<i>gap</i>	Glyceraldehyde-3-phosphate dehydrogenase	1.32	1.98	1.13	0.90
00796	<i>pgk</i>	Phosphoglycerate kinase	0.87	2.13	0.72	1.01
00798	<i>pgm</i>	2,3-Diphosphoglycerate-independent phosphoglycerate mutase	0.54	1.81	0.50	0.98
00799	<i>eno</i>	Enolase	0.92	1.15	0.88	1.06
01806	<i>pykA</i>	Pyruvate kinase	0.97	1.01	0.94	0.99
01041	<i>pdhB</i>	Pyruvate dehydrogenase E1	0.79	0.56	0.58	1.01
01043	<i>pdhD</i>	Dihydrolipoamide dehydrogenase E3	0.72	0.49	0.58	0.98
01042	<i>pdhC</i>	Dihydrolipoamide S-acetyltransferase E2	0.79	0.58	0.65	1.02

^a S refers to RN4220spacmvaS, A refers to RN4220spacmvaA, K refers to RN4220spack, and WT refers to RN4220. "+" refers to cells grown in the presence of 1 mM IPTG; "-" refers to cells grown in the absence of IPTG.

TABLE 7. Transcriptional changes in autolysis

RN4220 ORF (SAOUHSC no.)	Gene	Description	Fold change ^a			
			S-/S+	A-/A+	K-/K+	WT-/WT+
00994	<i>atl</i>	Bifunctional autolysin	0.59	0.42	0.31	0.87
00427		<i>N</i> -Acetylmuramoyl-L-alanine amidase	0.47	0.66	0.31	0.86
00671		Secretory antigen SsaA homologue	0.75	0.85	0.66	1.01
01219	<i>lytN</i>	Cell wall hydrolase	0.96	0.65	0.62	1.04
00998	<i>fnt</i>	Autolysis and methicillin resistant-related protein	2.50	3.77	2.38	0.93

^a S refers to RN4220spacmvaS, A refers to RN4220spacmvaA, K refers to RN4220spacK, and WT refers to RN4220. “+” refers to cells grown in the presence of 1 mM IPTG; “-” refers to cells grown in the absence of IPTG.

data in Table 7 demonstrate that there is reduced expression of several autolytic enzymes, including *atl* and *lytN*, as well as a >2-fold upregulation in the expression of *fnt*, an autolysis resistance-related protein, in response to *mvaS*, *mvaA*, or *mvaK* downregulation. Figure 3 shows that the downregulated mutants indeed have substantially decreased rates of autolysis in the Triton X-100 autolysis assay compared to wild-type RN4220 *S. aureus* and IPTG-induced RN4220spacmvaA, RN4220spacmvaS, and RN4220spacmvaK.

DISCUSSION

Isoprenoids, a class of ubiquitous organic compounds derived from the five-carbon starter unit IPP, have been intensely investigated for decades. Since 1956, when mevalonate was first discovered to be precursor to cholesterol, and continuing through the 1960s when the mevalonate pathway was fully elucidated (12), isoprenoid biosynthesis in mammalian cells has served as an important area for therapeutic intervention. The importance of the mevalonate pathway in animal cells is underscored by the use of farnesyl pyrophosphate in biosynthesis of dolichol, heme A, ubiquinone, and farnesylated proteins, and the use of cholesterol in the biosynthesis of steroid hormones, vitamin D, bile acids, and lipoproteins (7). However, despite the intense effort put forth into understanding the mevalonate pathway in eukaryotic cells, very little is known about the regulation of bacterial isoprenoid biosynthesis.

In the present study, to investigate the bacterial response to perturbations in the mevalonate pathway, *S. aureus* strains were constructed in which *mvaS*, *mvaA*, or *mvaK1/D/K2* were

placed under the control of the IPTG-inducible Pspac promoter. Characterization of these strains confirms the essentiality of these genes to bacterial cells, since the lack of expression of any of the pathway genes resulted in a dramatic reduction in bacterial growth rate, a defect that could be rescued with chemical complementation of mevalonate for the strains downregulated in *mvaS* or *mvaA*. These results are consistent with previous findings showing that all five genes encoding enzymes in the mevalonate pathway are essential for the growth of *S. pneumoniae* (28).

Our profiling results demonstrated that the transcriptome of *S. aureus* responds similarly to downregulation of either *mvaS*, *mvaA*, or *mvaK1/D/K2*, with 224 upregulated and 180 downregulated genes common to all three uninduced mutants. Many of the upregulated genes were found to be involved in stress response and virulence and those which were downregulated were mainly involved in primary metabolism, including nucleotide, heme, and protein biosynthesis, β -oxidation, and respiration. Such a dramatic shift in the transcriptome of *S. aureus* may be indicative of an altered metabolic state, where growth is halted and a defensive posture is assumed.

Given that undecaprenyl pyrophosphate is the key carrier lipid in peptidoglycan biosynthesis, it was not surprising that suboptimal levels of isoprenoids had a significant effect on cell wall biosynthesis. Inhibition of the mevalonate pathway at any step caused complete and robust activation of the cell wall stimulon, a group of genes defined by their collective response to several cell wall targeting antibiotics (11, 15, 16, 26). This transcriptional change was accompanied by a corresponding change in metabolism in *S. aureus*. Based on the GP2 microplate metabolic footprinting assay, cells deficient in IPP biosynthesis gained the ability to ferment several carbon sources which are precursor to the cell wall, perhaps in an attempt to compensate for deficiencies in peptidoglycan biosynthesis. Furthermore, inhibition of the mevalonate pathway genes robustly reduced the rate of autolysis in *S. aureus*. These results were consistent with previous data that demonstrated reciprocal coregulation of cell wall synthetic and hydrolytic enzymes in *S. aureus* (1).

Although downregulation of the mevalonate pathway clearly inhibited cell wall biosynthesis, this inhibition cannot explain all of the transcriptional changes observed. If all 458 genes found to be upregulated in at least one of the studies conducted to define the cell wall stimulon (11, 15, 16, 26) are compared to the 224 genes found to be upregulated in all three mevalonate pathway downregulated mutants, only 74 common genes emerge. Furthermore, 33 genes found to be upregulated

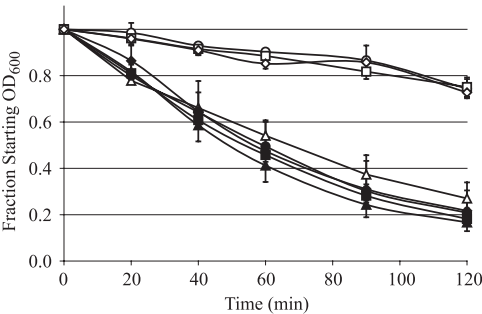


FIG. 3. Triton X-100 induced autolysis of RN4220 (triangles), RN4220spacmvaS (diamonds), RN4220spacmvaA (circles), and RN4220spacmvaK (squares) that have grown with (filled) or without (unfilled) 1 mM IPTG induction. Bars represent the standard deviation of four independent experiments.

during treatment with at least one cell wall targeting antibiotic are actually downregulated in response to mevalonate pathway inhibition. These data demonstrate that downregulation of the mevalonate pathway produces changes that are unique and different compared to the inhibition of cell wall biosynthesis alone.

Perhaps most interestingly, inhibition of *mvaS*, *mvaA*, or *mvaK1/D/K2* elicited very little compensatory transcription of other isoprenoid biosynthetic genes. The largest changes were a twofold upregulation of the *mvaK1/D/K2* operon, a twofold upregulation of *upps* and *bacA*, and a twofold downregulation of the *crt* operon. The downregulation of staphyloxanthin biosynthetic genes and the upregulation of undecaprenyl diphosphate synthase may be indicative of *S. aureus* attempting to divert limited IPP precursor from nonessential to essential metabolites. Most notably, regulation in the early steps of mevalonate biosynthesis appears to occur at the level of *mvaK1/D/K2*, which is in contrast to regulation in eukaryotes which occurs at the level of *mvaA* and *mvaS*. Studies have shown that sterol regulatory element 1 upstream of the promoters for HMG-CoA synthase and HMG-CoA reductase allows for a positive response in the absence of sterols (7). Here we demonstrate both with transcriptional profiling and qRT-PCR that limiting quantities of isoprenoids have little effect on the transcriptional levels of *mvaS* and *mvaA* but induce transcription of *mvaK1/D/K2*.

Although transcriptional regulation of the mevalonate pathway occurs at the level of *mvaK1/D/K2*, *mvaA* clearly plays an important regulatory role in the cell, since its downregulation incurred more and specific changes not observed when downregulating the other mevalonate pathway genes. In particular, underexpression of *mvaA* appears to directly affect glycolysis, both increasing the transcription of genes involved in the process and increasing the utilization of substrates that feed into the pathway. Metabolic footprinting revealed that only in the *mvaA* downregulated strain did *S. aureus* gain the ability to utilize mannitol, D-fructose-6-phosphate, and D-glucose-6-phosphate, three carbon sources that are direct substrates of the first three steps in glycolysis. This altered fermentation pattern correlated directly with upregulation of four intermediate genes in glycolysis: triose phosphate isomerase, glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase, and phosphoglycerate mutase. Furthermore, when we analyzed other transcriptional changes specific to the *mvaA* mutant, we found that three genes—glycerol uptake facilitator, glycerol kinase, and glycerol-3-phosphate dehydrogenase—responsible for metabolizing glycerol to dihydroxyacetone phosphate, an intermediate in glycolysis, are upregulated. Since glycolysis is responsible for the generation of acetyl-CoA, the sole precursor of the mevalonate pathway, increased glycolytic activity may be a response to the accumulation of HMG-CoA or the absence of mevalonate caused by loss of MvaA activity. Interestingly, Table 6 also shows that the three components of the pyruvate dehydrogenase complex are downregulated ~2-fold in the *mvaA* mutant, which could be a secondary response to the buildup of acetyl-CoA initially synthesized to deal with stalled mevalonate biosynthesis. In any event, *mvaA* appears to be tied in with key sensory functions of the cell. The unique importance of *mvaA* has been previously observed in *S. pneumoniae*, where *mvaA* cannot be knocked

out independently of *mvaS* even when the desired auxotroph is supplemented with exogenous mevalonate (28).

Overall, the large number of genes with altered expression is demonstrative of the complexity of feedback mechanisms connected to isoprenoid biosynthesis present in *S. aureus*. Inhibition of isoprenoid biosynthesis has massive consequences on cell wall biosynthesis, affecting cell viability, primary metabolism, and fermentative capacity. Although transcriptional regulation of the mevalonate pathway appears to occur at the level of *mvaK1/D/K2*, *mvaA* clearly plays a crucial role in how *S. aureus* perceives and responds to inhibition of this essential pathway.

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