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Identification, Evolution, and Essentiality of the Mevalonate Pathway for Isopentenyl Diphosphate Biosynthesis in Gram-Positive Cocci

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The mevalonate pathway and the glyceraldehyde 3-phosphate (GAP)–pyruvate pathway are alternative routes for the biosynthesis of the central isoprenoid precursor, isopentenyl diphosphate. Genomic analysis revealed that the staphylococci, streptococci, and enterococci possess genes predicted to encode all of the enzymes of the mevalonate pathway and not the GAP-pyruvate pathway, unlike *Bacillus subtilis* and most gram-negative bacteria studied, which possess only components of the latter pathway. Phylogenetic and comparative genome analyses suggest that the genes for mevalonate biosynthesis in gram-positive cocci, which are highly divergent from those of mammals, were horizontally transferred from a primitive eukaryotic cell. Enterococci uniquely encode a bifunctional protein predicted to possess both 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and acetyl-CoA acetyltransferase activities. Genetic disruption experiments have shown that five genes encoding proteins involved in this pathway (HMG-CoA synthase, HMG-CoA reductase, mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase) are essential for the in vitro growth of *Streptococcus pneumoniae* under standard conditions. Allelic replacement of the HMG-CoA synthase gene rendered the organism auxotrophic for mevalonate and severely attenuated in a murine respiratory tract infection model. The mevalonate pathway thus represents a potential antibacterial target in the low-G+C gram-positive cocci.

Isoprenoids, which are ubiquitous in nature, comprise a family of over 23,000 products, each composed of repeating five carbon isopentenyl diphosphate (IPP) subunits. Among the isoprenoids are sterols, which contribute to eukaryotic membrane architecture, steroid hormones, chlorophylls, and the acyclic polyprenoid plastoquinone (42). In bacteria, the principal products of IPP include the lipid carrier undecaprenol which is involved in cell wall biosynthesis (36), menaquinones and ubiquinones involved in electron transport (32), and carotenoids (23).

Two pathways for the biosynthesis of IPP have been described, the classical mevalonate pathway and the more recently identified glyceraldehyde 3-phosphate (GAP)–pyruvate pathway. Rohmer et al. (38) demonstrated the presence of the non-mevalonate pathway, originating from pyruvate and GAP, in several gram-negative bacteria, including *Escherichia coli*. The GAP-pyruvate pathway is also present in *Chlamydia trachomatis* (44) and *Aquifex aeolicus* (13), the gram-positive bacteria *Bacillus subtilis* (52) and *Mycobacterium tuberculosis* (35), the cyanobacterium *Synechocystis* (14), chloroplasts (29), and some unicellular algae, including *Scenedesmus oliquus* (40).

The mevalonate pathway for IPP biosynthesis was first identified in eukaryotic cells (17). In this pathway (Fig. 1), three acetyl coenzyme A (acetyl-CoA) units are joined successively to form 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). HMG-CoA is then reduced to mevalonate, which is subsequently phosphorylated, decarboxylated, and dehydrated to form IPP. The enzymes of the mevalonate pathway from a number of organisms, including humans, have been studied. HMG-CoA reductase, the best-characterized enzyme in the pathway, is the target of the statin class of cholesterol-lowering drugs (1). Particularly well characterized is the biodegradative HMG-CoA reductase from *Pseudomonas mevalonii*, which catalyzes the conversion of mevalonate to HMG-CoA, enabling the organism to use mevalonate as its sole source of carbon (4). *P. mevalonii*, however, does not appear to possess the other enzymes of the mevalonate pathway and hence probably synthesizes IPP via the GAP-pyruvate pathway.

While the genome of *B. subtilis* revealed genes encoding enzymes of the GAP-pyruvate pathway for IPP biosynthesis, other gram-positive bacteria, including *Lactobacillus plantarum* (49), *Staphylococcus aureus* (18, 33), *Staphylococcus carnosus* (21), and *Streptococcus mutans* (50), incorporate radiolabeled mevalonate into isoprenoids, suggesting that these bacteria use the mevalonate pathway for the synthesis of IPP. Furthermore, Doolittle and Lodgson (15) and Bochar et al. (7) reported the presence of the HMG-CoA reductase gene in the genomes of *Streptococcus pneumoniae* and *Streptococcus pyogenes*. The majority of *Streptomyces* species are reported to possess only the GAP-pyruvate pathway, but some species, such as *Streptomyces aeriouvifer*, possess both pathways for the synthesis of isoprenoids (41), perhaps accounting for the abundance of isoprenoid compounds produced as low-molecular-weight secondary metabolites. Among microorganisms there is genomic and/or biochemical evidence to suggest the presence of the mevalonate pathway in the spirochete *Borrelia burgdorferi* (AE001169), the gram-negative bacteria *Chloropseudomonas ethylica* and *Myxococcus fulvus* (21), the archaea (5, 6), fungi (28), and species of algae such as *Euglena gracilis* (14, 43).

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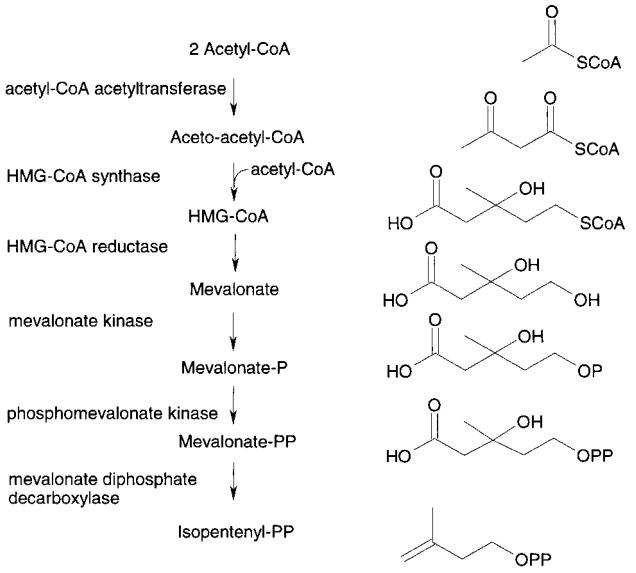

FIG. 1. Biosynthesis of IPP via the mevalonate pathway.

We report here that, unlike most gram-negative bacteria and *B. subtilis*, species of enterococci, staphylococci, and streptococci possess homologs of five genes predicted to encode enzymes involved in the mevalonate route to IPP but not homologs of the genes involved in the GAP-pyruvate pathway. Phylogenetic analysis suggests that gram-positive cocci obtained the genes for the mevalonate pathway through one or more horizontal gene transfer events involving a primitive eukaryote. The genes are arranged in one or two clusters. In all species, the HMG-CoA synthase and HMG-CoA reductase genes are closely linked but are divergently transcribed in the enterococci and staphylococci. The mevalonate kinase, mevalonate decarboxylase, and phosphomevalonate kinase genes constitute an apparent single operon, whose structure is highly conserved in the species examined. The enterococci appear to encode a bifunctional protein predicted to possess both HMG-CoA reductase and acetyl-CoA acetyltransferase (thiolase) activities. Using allelic replacement techniques, we have demonstrated that each of the five genes of the mevalonate pathway is essential for the growth of *Streptococcus pneumoniae* in vitro without added mevalonate.

MATERIALS AND METHODS

Identification and phylogenetic analysis of mevalonate pathway gene sequences. Separate database searches and phylogenetic analyses were performed for the five proteins MvaS, MvaA, MvaK1, MvaK2, and MvaD. Homologous protein sequences were retrieved from public and proprietary genomic sequence databases including the Incyte Pharmaceutical Inc. PathoSeq sequence database by using BLASTP and TBLASTN software (2). Preliminary sequence data were also obtained from The Institute for Genomic Research website at http://www.tigr.org. The proteins were aligned using the program CLUSTALW version 1.7 (48) with the BLOSUM62 (20) similarity matrix and gap opening and extension penalties of 10.0 and 0.05, respectively. The multiple-sequence alignments were refined manually using the program SEQLAB of the GCG version 9.0 software package (Genetics Computer Group, Madison, Wis.).

Phylogenetic trees were constructed by maximum-parsimony (MP) and neighbor-joining (NJ) methods for each set of alignments. NJ trees were based on pairwise distances between amino acid sequences using the programs NEIGHBOR and PROTDIST of the PHYLIP 3.57c package (http://evolution.genetics.washington.edu/phylip.html) (16). The Dayhoff program option was invoked in the latter program, which estimates based on the Dayhoff 120 matrix. The programs SEQBOOT and CONSENSE were used to estimate the confidence limits of branching points from 1,000 bootstrap replications. MP analysis was done using the software package PAUP* (45). Given the large size of the data set, it was not possible to exhaustively search for the total number of minimal-length trees. Instead, the numbers and lengths of minimal trees were estimated from 100 replicate random heuristic searches while confidence limits of branch points were estimated by 1,000 bootstrap replications.

Bacterial strains, media, plasmid, enzymes and chemicals. *S. pneumoniae* R6 was as reported by Avery et al. (3); *S. pneumoniae* 0100993 was a clinical isolate kindly provided by D. Felmington (University College Hospital, London, United Kingdom); *Enterococcus faecium* 2, *Staphylococcus haemolyticus* 225, and *Staphylococcus epidermidis* CL-7 were clinical isolates from the SmithKline Beecham culture collection. *E. faecium* was grown in Luria-Bertani broth (39). *S. haemolyticus* and *S. epidermidis* were grown on tryptic soy broth (TSB) and agar (TSA) (Oxoid). pAMβ1 was as reported by Martin et al. (31). Chromosomal DNA was prepared using published procedures (30), except that 0.1 μg of lysostaphin per ml (Applied Microbiology Inc.) was included for the lysis of *S. epidermidis* and *S. haemolyticus*. Chromosomal DNA was amplified using *PfuTurbo* DNA polymerase (Stratagene) under conditions recommended by the manufacturer. Restriction enzymes were obtained from GibcoBRL and used as specified by the manufacturer. All chemicals were from Sigma Chemical Co., unless otherwise specified.

Completion of nucleotide sequences. Analysis of sequence databases revealed gaps in the nucleotide sequences of *mvaA*, *mvaK1*, *mvaK2*, and *mvaD* from *E. faecium* and *mvaK2* from *S. epidermidis* and *S. haemolyticus*. Gaps were completed by comparing the operon structure from other members of the genus, designing primers, and amplifying the missing sequence. PCR products were sequenced directly on both strands. For *mvaK1* of *E. faecium*, chromosomal DNA was sequenced directly to complete the 5′ end of the gene. Nucleotide and amino acid sequences were analyzed with the GCG version 9.0 and Lasergene (DNASTAR, Madison, Wis.) software packages.

Generation of *S. pneumoniae* allelic replacement mutants. Chromosomal DNA fragments (500 bp long) flanking the genes of interest were PCR amplified from *S. pneumoniae* 0100993 chromosomal DNA. Primers were designed so that flanking genes and potential promoters would remain intact in the deletion mutant to minimize polar effects. The fragments were used to make constructs in which they flanked the erythromycin resistance gene *ermAM* from pAMβ1. A total of 10^6 *S. pneumoniae* R6 competent cells prepared by published methods were incubated with 500 ng of allelic replacement construct at 30°C for 30 min and transferred to 37°C for 90 min to allow expression of antibiotic resistance. The transformation mixes were plated in AGCH agar (24) containing 1 μg of erythromycin per ml and incubated at 37°C for 36 h under 5% CO_2. Where appropriate, transformation and growth media were supplemented with 10 mM mevalonate. If no transformants were obtained in three separate transformation experiments with positive allelic replacement and transformation controls, the target gene was considered to be essential in vitro under the conditions chosen.

Erythromycin-resistant *S. pneumoniae* R6 colonies were picked and grown overnight in Todd-Hewitt broth (Difco) supplemented with 5% (wt/vol) yeast extract. Chromosomal DNA was prepared and used to transform *S. pneumoniae* 0100993 in the presence of 1 μg of competence-stimulating heptadecapeptide per ml (19). Chromosomal DNA from *S. pneumoniae* 0100993 Erm^r clones was examined using Southern blot analysis and diagnostic PCR to verify that the appropriate chromosomal DNA rearrangement had occurred. In the former, flanking DNA fragments labeled using the ECL random-prime labeling kit (Amersham Pharmacia Biotech) were used as probes to chromosomal DNA restricted with appropriate enzymes and blotted using standard methods (39). In the latter, DNA primers designed to hybridize within *ermAM* were paired with primers hybridizing to distal chromosomal sequences to generate DNA amplification products of characteristic sizes.

Mouse respiratory tract infection model using *S. pneumoniae*. *S. pneumoniae* 0100993 and the *mvaS* null mutant were grown on TSA plates containing 5% (vol/vol) sheep blood (BBL) (supplemented with 10 mM mevalonate as appropriate) at 37°C in 5% CO_2 overnight. Bacteria were recovered from the plates and resuspended in phosphate-buffered saline to an absorbance at 600 nm of ~0.9 (~10^7 CFU ml^{-1}). For each strain used, five male CBA/J mice (14 to 16 g) were anesthetized with 3% isoflurane and 50 μl of inoculum was administered by intranasal instillation. The mice were given food and water ad libitum. Mice were sacrificed at 12 or 48 h postinoculation by CO_2 overdose, their lungs were aseptically removed and homogenized in 1 ml of phosphate-buffered saline, and viable bacteria were enumerated by serial dilution and viable counting on blood agar plates supplemented with 10 mM mevalonate for the *mvaS* null mutant.

RESULTS

Identification of mevalonate pathway genes in gram-positive cocci. Through genomic analysis, the five genes encoding enzymes involved in the mevalonate pathway (Fig. 1) were identified in the low-G+C gram-positive cocci *S. aureus*, *S. epidermidis*, *S. haemolyticus*, *S. pyogenes*, *S. pneumoniae*, *E. faecalis*, and *E. faecium*. Primers for amplification of missing gene sequences were designed by comparing the gene arrangements in these related species, and additional sequencing resulted in the

completion of the full-length gene sequences. An extensive homology search of sequence databases comprising over 60 partial and complete bacterial genomes did not reveal the presence of the complete set of mevalonate pathway genes in bacteria other than the low-G+C gram-positive cocci and *B. burgdorferi*. With the exception of the recently discovered *ygbP* gene, which encodes 4-diphosphocytidyl-2-*C*-methylerythritol synthase (37), genomes of the gram-positive cocci do not possess genes predicted to encode enzymes involved in the GAP-pyruvate pathway, although the genomes are incomplete. Only a single copy of each gene was identified in each species, except for the first enzyme in the pathway (acetyl-CoA acetyltransferase). Although three homologs were found in *S. pyogenes*, no putative acetyl-CoA acetyltransferase homologs were identified in the partial genomes of four strains of *S. pneumoniae*. A single open reading frame (*mvaE*) predicted to encode a single polypeptide with both acetyl-CoA acetyltransferase and HMG-CoA reductase activities was identified in *E. faecalis* and *E. faecium*. The amino-terminal region of the *E. faecalis* enzyme was 48% identical to the full length acetyl-CoA acetyltransferase enzyme from *Thermoanaerobacterium thermosaccharolyticum* (Z82038.1), and the carboxy-terminal end was 42% identical to the full length HMG-CoA reductase enzyme from *Archaeoglobus fulgidus* (AE000983.1). Sequence alignment programs and visual inspection were employed to compare the derived amino acid sequences from the five enzymes involved in the mevalonate pathway. Amino acid residues identified as important in catalysis in homologous eukaryotic, eubacterial, and archaeal enzymes are conserved in the enzymes of the gram-positive bacteria (Fig. 2). Multiple sequence alignments are available upon request from the authors (James_R_Brown@sbphrd.com).

Phylogenetic analysis. MP and NJ methods showed similar overall tree topologies for the four enzyme families (Fig. 3). Each of the phylogenetic analyses showed strong statistical support, in terms of bootstrap values and minimal-length trees, for clustering the enzymes from gram-positive bacteria together. Phylogenetic analyses were based on the total number of sites in edited multiple-sequence alignments for the proteins HMG-CoA synthase (275 sites), HMG-CoA reductase (331 sites), mevalonate and phosphomevalonate kinase (184 sites), and mevalonate diphosphate decarboxylase (292 sites). The clusters of gram-positive bacterial enzymes showed high levels of sequence identity (based on the length of the shorter sequence without gaps from the edited alignment) for each of the five enzymes HMG-CoA synthase (48 to 90%), HMG-CoA reductase (45 to 90%), mevalonate kinase (39 to 81%), phosphomevalonate kinase (34 to 87%), and mevalonate diphosphate decarboxylase (39 to 80%). Single minimal-length MP trees were determined for HMG-CoA synthase (minimal length [ml] = 1,617 steps) and mevalonate diphosphate decarboxylase (ml = 1,177 steps). For the mevalonate kinase family, two MP trees were found (ml = 1,812 steps), one of which clustered eukaryotes and gram-positive cocci together and one of which grouped the eukaryotes with the archaea. Sixteen MP trees were found for HMG-CoA reductase (ml = 3,430 steps) which differed only in the arrangements of the terminal class I taxa.

Although all protein trees are unrooted, the gram-positive bacterial mevalonate pathway enzymes are generally most similar to eukaryotic versions with the exception of HMG-CoA reductase, where the bacteria have enzymes of the class II rather than class I type (7). In the HMG-CoA synthase tree, there are three major clusters, the archaea, bacteria, and eukaryotes, and the tree could be rooted in any branch, although overall sequence similarities are slightly greater between eukaryotes and bacteria. Two distinct types of kinase were found. The mevalonate kinase gene (*mvaK1*) occurs in the archaea, bacteria, and eukaryotes, while the phosphomevalonate kinase gene (*mvaK2*) is restricted to gram-positive cocci, *B. burgdorferi*, and *Saccharomyces cerevisiae*. Phylogenetic analysis suggests that phosphomevalonate kinases are an ancient group of proteins and did not arise from gene duplications within the gram-positive cocci. The branching order of archaeal, eukaryotic, and bacterial *mvaK1* could not be resolved, mainly because the archaea were not monophyletic. Extensive homology searches failed to detect the *mvaD* gene, encoding mevalonate diphosphate decarboxylase, in any archaeal genomes, although this enzyme is present in eukaryotes, gram-positive cocci, and *B. burgdorferi*.

Gene organization of the mevalonate pathway. The mevalonate pathway genes are located at two positions on the chromosome in the gram-positive bacteria examined, except in *S. pyogenes*, where all five genes are clustered at a single location (Fig. 4). The organization of the *mvaK1DK2* genes is identical in the gram-positive cocci. A fourth gene (*ypgA*) is present downstream of *mvaK2* in the enterococci and streptococci. Although its function is unknown, *ypgA* is also part of the mevalonate pathway operon in *B. burgdorferi*, and in *Erwinia herbicola* (open reading frame 6) it is clustered with genes involved in carotenoid biosynthesis (22), rather than with mevalonate pathway genes. *Erwinia* species are reported to possess the GAP-pyruvate pathway for IPP biosynthesis (35). A copy of *ypgA* is present in the staphylococci but at a different location on the chromosome. The organization of the HMG-CoA synthase (*mvaS*) and reductase (*mvaA*) genes is less well conserved, although in all cases they are either adjacent to each other or separated by an acetyl-CoA acetyltransferase (*mvaC*) coding region. *mvaS* and *mvaA* are divergently transcribed in the staphylococci and enterococci but transcribed in the same direction in the streptococci.

Essentiality of the mevalonate pathway genes in *S. pneumoniae* R6. DNA constructs containing an erythromycin resistance gene flanked by DNA adjacent to the target gene were generated for the allelic replacement of *mvaS*, *mvaA*, *mvaK1*, *mvaK2*, and *mvaD* and used to transform *S. pneumoniae* R6. It was not possible to obtain allelic exchange mutants for any of the genes in the absence of added mevalonate, suggesting that all of the genes are essential for the in vitro growth of *S. pneumoniae* under the standard growth conditions.

It was predicted from the pathway that mutants auxotrophic for mevalonate could be obtained. HMG-CoA, mevalonate phosphate, mevalonate diphosphate, and IPP were not expected to penetrate the cell and were not included in complementation studies. When the transformation and growth media were supplemented with 10 mM mevalonate, *mvaS* auxotrophic mutants were readily obtained (data not shown), and the allelic replacement was confirmed using Southern blot analysis and diagnostic PCR (Fig. 5). Surprisingly, it was not possible to obtain a *mvaA* auxotrophic mutant in multiple experiments under similar conditions, although double allelic replacement of both *mvaA* and *mvaS* was readily achieved and these mutants were auxotrophic for mevalonate.

Investigation of mevalonate auxotrophy of the *mvaS* null mutant. A minimum concentration of 3 mM mevalonate was required by the *mvaS* null mutant for growth in Todd-Hewitt broth plus 5% (wt/vol) yeast extract (data not shown). An overnight culture of the *mvaS* null mutant grown in the presence of 3 mM mevalonate was diluted (10^4-fold) into fresh medium with and without mevalonate, and the number of viable cells over 8 h was determined by plating on medium containing mevalonate (data not shown). In the unsupple-

HMG-CoA synthase

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EFA  99 QPFARSFEIKEACYGATAGLQLAK   230 LAFHIPYTKM   274 GNIYTGSLYLGLISLLEN--ATTLTAGNQIGLFSYGSGAV
EFM  99 QSFARSFEVKEACYGGTAALHMAK   230 IAFHIPYTKM   274 GNLYTGSLYLGLTSLLEN--SKSLQPGDRIGLFSYGSGAV
SEP  99 QPFARCFEMKEACYAATPAIQLAK   230 LCFHVPFTKM   274 GNIYTGSLYLSLISLLET---RDLKGGQTIGLFSYGSGSV
SHA  99 QPFARCFEMKEACYAATPAIQLAK   230 LCFHVPFTKM   274 GNIYTGSLYLSLISLLEN---RSLKGGQTIGLFSYGSGSV
SAU  99 QPFARCFEMKEACYAATPAIQLAK   230 LCFHVPFTKM   274 GNIYTGSLYLSLISLLEN---RDLQAGETIGLFSYGSGSV
SCA  99 QPFARCFEMKEACYAATPAIQLAK   230 LCFHVPFTKM   275 GNVYTGSLYLSLISLLEN---HKLNGGDNIGLFSYGSGSV
SPN  99 QPFARSFEIKEACYGATAALHYAK   230 VCFHLPYPKL   275 GNIYTGSLFLGLLSLLEN--TDSLKAGDKIALYSYGSGAV
SPY  99 QPFARSFEVKQACYSATAALDYAK   230 VCFHIPFPKL   275 GNIYTGSLYLGLLSLLEN--SKVLQSGDKIGFFSYGSGAV
BBO 115 NNFLT-FQVQHACAGAAMSLHTVA   261 FVLHVPFAKM   272 GNLYTGSIFLSLAFYLKRVFSKKDITGEKILFCSYGSGNI
HUM 114 NTDIEGIDTTNACYGGTAAVFNAV   261 MIFHSPYCKL   342 GNMYTSSVYGSLASVLAQYSPQQL-AGKRIGVFSYGSGLA
MIT 154 NTDIEGIDTTNACYGGTASLFNAA   298 MIFHTPFCKL   379 GNMYTSSLYGCLASLLSQCSAQDL-AGSRIGAFSYGSGLA
SAC 147 NTDVEGIDTLNACYGGTNALFNSL   293 NVFHVPTCKL   370 GNMYTASVYAAFASLLNYVGSDDL-KGKRVGAYSYGSGLA
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HMG-CoA reductase (Class II)

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EFA 435 ENQISETEVPMGVGLHLTVDETDYLVPMATEEPSVIAALSNGAKI   559 VDVKDAMAGNI   637 RAVTHNKGIMNGIEAVVLATGNDTR   697 PLALATVGGAT   746 ALVSEGIQKGHMAL
EFM 441 ENQVSEVEIPMGIAQNFQINGKKKWIPMATEEPSVIAAASNGAKI   568 VDVKDAMGANM   647 RAATHNKGIMNGIEAVVAATGNDTR   707 PLAVATVGGAS   756 ALVTEGIQKGHMGL
SEP  50 ENVIGQGALPVGLLPRIIVDDKEYVVPMMVEEPSVVAAASYGAKL   178 VDTKDAMGANM   257 RAATHNKGVMNGIHAVVLATGNDTR   319 PMTLATVGGGT   368 ALVSEGIQQGHMSL
SHA  50 ENVIGQGTLPVGLLPKIIVDDKEYVVPMMVEEPSVVAAASYGAKL   178 VDTKDAMGANM   257 RAATHNKGVMNGIHAVVLATGNDTR   319 PMTLAIVGGGT   368 ALVSEGIQKGHMSL
SAU  50 ENVIAQGALPVGLLPNIIVDDKAYVVPMMVEEPSVVAAASYGAKL   178 VDTKDAMGANM   257 RAATHNKGVMNGIHAVVLATGNDTR   319 PMTLAIVGGGT   368 ALVSEGIQQGHMSL
SPN  50 ENVVGTFSLPYSLVPEVLVNGQEYTVPYVTEEPSVVAAASYASKI   179 VDTQEAMGANM   257 RAATHNKGIFNGIDAILIATGNDWR   319 PMPVATKGGSI   368 ALVSTGIQQGHMKL
SPY  53 ENVLGRLALPFSIAPDFLVNGSTYQMPFVTEEPSVVAAASFAAKL   179 VDTQEAMGANM   257 RATTHNKGIFNGIDAVVIATGNDWR   319 PMPIASKGGSI   368 ALTSSGIQAGHMKL
BBO  54 ENYIGYLSFPIGIVKNLKINGKYYSLPIATEESSVVAALNFAAKI   178 VDTCDAMGANL   256 RAVTNNKGIMNGITGVCLATFNDTR   316 PLQVGTKGGVI   365 ALAFNGIQKGHMRL
PME  52 ENVIGTFELPYAVASNFQINGRDVLVPLVVEEPSIVAAASYMAKL   183 VDVRDAMGANT   261 RAATHNKGIMNGIDPLIVATGNDWR   322 PMPVGLVGGAT   371 ALATEGIQRGHMAL
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Mevalonate kinase

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EFA 14 KIILMGEHAVVYGEPAIAFP    96 IPAERGMGSSAATAVAIVR   137 EKIAHGNPSGIDA   265 GALGAKLTGGGRGGCMIAL
EFM 12 KIILMGEHAVVYGEPAIAFP    94 IPAERGMGSSAAVATAVTR   135 ERMIHGNPSGVDN   262 GALGAKLTGGGRGGCMIAL
SEP 12 KIILMGEHAVTFGQPAIAIP    93 LPPSRGLGSSAAVAVAFVR   134 EQIAHGKPSGIDT   259 GAIAGKLTGGGRGGSMLLL
SHA 12 KIILIGEHAVTFGEPAIAIP    93 LPPSRGLGSSAAVAVAFIR   134 ERIAHGKPSGIDT   260 GSVAGKLTGGGRGGSMLIL
SAU 12 KIILIGEHAVTFGEPAIAVP    93 LPPSRGLGSSAAVAVAFVR   134 EQIAHGKPSGIDT   259 GAIAGKLTGAGRGGSMLLL
SPN 13 KIILIGEHAVVYGYPAISLP    83 IPEKRGMGSSAAISIAAIR   114 EMIAHMNPSGLDA   247 GALGAKMSGGGLGGCIIAL
SPY 13 KIILIGEHAVVYGYPAIALP    83 VPQKRGMGSSAAVSIAAIR   114 EIIAHTNPSGLDA   247 GALGAKMTGGGLGGCMIAL
BBO 11 KILFLGEHSAVYGFPVIG--    78 IPIGVGLGSSASLSLCFAE   117 ENIFHGKSSGMDI   245 GALGAKLTGGGRGGCMIAL
HUM 13 KVILHGEHAVVHGKVALAVS   137 LPPGAGLGSSAAYSVCLAA   193 ERMIHGNPSGVDN   325 RGLHSKLTGAGGGGCGITL
SAC 12 KVIIFGEHSAVYNKPAVAAS   140 LPIGAGLGSSASISVSLAL   191 EKCIHGTPSGIDN   343 RIGSTKLTGAGGGGCSLTL
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Phosphomevalonate kinase

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EFA 5 TTPGKLFIAGEYAVVEPG   111 NGRKYGLGSSGAVTVGTVKAL
EFM 5 SAPGKLYIAGEYAVVETG   111 NGRKYGLGSSGAVTVATVKAL
SEP 5 KAPGKLYIAGEYAVTEPG   114 SGQKYGLGSSAAVLVSVVKAL
SHA 5 KAPGKLYVAGEYAVTEPG   114 SGNKYGLGSSAAVLVSVVKAL
SAU 5 KAPGKLYIAGEYAVTEPG   114 NGHKYGLGSSAAVLVSVIKVL
SPN 5 KTCGKLYWAGEYAILEPG    98 EGKKFGLGSSGSVVVLVVKAL
SPY 7 QTGGKLYLTGEYAILIPG   100 DGKKFGIGSSGSVTLLTLKAL
BBO 7 SVPGNLLLMGEYTILEEK   101 DGTKKGFGSSAVVAIGIVCGL
SAC 8 SAPGKALLAGGYLVLDTK   149 EVPKTGLGSSAGLVTVLTTAL
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Mevalonate diphosphate decarboxylase

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EFA 11 NIALIKYWGKANEEYILPMNSSLSLTL    96 NFVPTAAGLASSASGLAALAGACNVALGLNLSAKDLSRLARRGSGSACRSIFGGFAQWNK   271 RAK--GIPCYFTMDAGPNVK
EFM 11 NIALIKYWGKKNEELILPMNNSLSLTL    96 NFVPTAAGLASSASGLAALAGACNTALKLGLDDLSLSRFARRGSGSACRSIFGGFVEWEK   271 RKQ--GIPCYFTMDAGPNVK
SEP 12 NIALIKYWGKADETYIIPMNNSLSVTL    97 NYVPTAAGLASSASAYAALAAACNEALSLNLSDTDLSRLARRGSGSASRSIFGGFAEWEK   272 RKA--NLPCYFTMDAGPNVK
SHA 12 NIALIKYWGKADEALIIPMNNSLSVTL    97 NFVPTAAGLASSASAYAALAGACNEALQLGLSDKDLSRLARRGSGSASRSIYGGFAEWEK   272 REA--GLPCYFTMDAGPNVK
SAU 12 NIALIKYWGKKDEALIIPMNNSISVTL    97 NFVPTAAGLASSASAYAALAAACNQALDLQLSDKDLSRLARIGSGSASRSIYGGFAEWEK   272 REA--GYPCYFTMDAGPNVK
SPN 13 NIAIIKYWGKKKEKEMVPATSSISLTL    96 NNMPTAAGLSSSSSGLSALVKACNQYFKLGLDRSQLAQEAKFASGSSSRSFYGPLGAWDK   265 REK--GEACYFTMDAGPNVK
SPY 13 NIAIIKYWGKENQAKMIPSTSSISLTL    96 NNMPTAAGLSSSSSGLSALVKACDQLFDTQLDQKALAQKAKFASGSSSRSFFGPVAAWDK   265 RQE--GFACYFTMDAGPNVK
BBO 10 SLALIKYWGKKDVFLNIPATSSLAVSV    90 NNFPTAAGLASSSSGFASIAACILKYFN-KYSCNSASNLARVGSASAARAIYGGFTILKE   259 RNE--GIFVFETMDAGPQVK
HUM 17 NIAVIKYWGKRDEELVLPINSSLSVTL   106 NNFPTAAGLASSAAGYACLAYTLARVYGVESD---LSEVARRGSGSACRSLYGGFVEWQM   292 NAHHGDTKVAYTFDAGPNAV
SAC 17 NIATLKYWGKRDTKLNLPTNSSISVTL   110 NNFPTAAGLASSASGYAALAGACNKALGLGLSDKDLSRLARRGSGSACRSIFGGFVEWEK   295 TIVAYTFDCYFTMDAGPNVK
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mented medium, the *mvaS* null mutant was unable to grow, although cells remained viable. The mutant's viable count increased by 3 log units over the same period in the presence of 3 mM mevalonate.

Virulence attenuation of the *mvaS* null mutant. The *mvaS* mutation was transferred to the pathogenic strain *S. pneumoniae* 0100993 by chromosomal transformation, and the mutants were auxotrophic for mevalonate. Mice were inoculated intranasally with either *S. pneumoniae* 0100993 or the *mvaS* null mutant. Viable *S. pneumoniae* 0100993 harboring the deletion could not be recovered from the lungs (i.e., below the limit of detection of 1.60 $\log_{10}$ CFU/mouse) after 12 or 48 h when plated in the presence of mevalonate, in contrast to the wild-type *S. pneumoniae* 0100993, which was present at 5.67 $\pm$ 0.11 and 7.39 $\pm$ 0.75 $\log_{10}$ CFU/mouse at 12 and 48 h, respectively (mean $\pm$ standard deviation for five mice). The results indicate that virulence in the mutant is severely attenuated at both early and late stages of respiratory tract infection.

DISCUSSION

The low-G+C gram-positive cocci are major pathogens, and novel targets for drug intervention are required to circumvent the resistance mechanisms which compromise current antibacterial agents. In a search for such novel drug targets, five full-length genes encoding enzymes that are predicted to catalyze consecutive steps in the mevalonate pathway for IPP biosynthesis were identified in seven species of gram-positive cocci. The nucleotide and derived amino acid sequences of HMG-CoA synthase, mevalonate kinase, phosphomevalonate kinase, and mevalonate diphosphate decarboxylase have not previously been identified in bacteria other than *Borrelia burgdorferi*, and the amino acid sequences of streptococcal biosynthetic HMG-CoA reductases have been reported only recently (7). None of the species possessed homologs of the genes identified in the GAP-pyruvate pathway for IPP synthesis other than *ygbP*, suggesting that isoprenoids are synthesized in the streptococci, staphylococci, and enterococci solely via the mevalonate pathway.

Based on amino acid sequence analysis, Bochar et al. (7) suggested that there are two distinct classes of HMG-CoA reductase: class I, found in most archaea, fungi, and eukaryotes and in *Streptomyces* CL190 (47), and class II, found in some bacteria and the archaeon *Archaeoglobus fulgidus*. HMG-CoA reductases identified in staphylococci, streptococci, and enterococci are of the class II type (Fig. 3) and show a high degree of sequence homology to the *Pseudomonas mevalonii* enzyme (Fig. 2). Residues involved in the catalysis of HMG-CoA reductases have been identified through crystallography (27, 46) and mutagenesis (12, 53), and these residues are conserved in the enzymes of the gram-positive cocci. The product of the *Staphylococcus aureus mvaA* gene has been isolated and shown to be an HMG-CoA reductase (unpublished results). The enterococci are uniquely predicted to synthesize a bifunctional protein which possesses both HMG-CoA reductase and acetyl-CoA acetyltransferase activities. These enzymes catalyze the first and third steps in the production of IPP, respectively (Fig. 1). No other genes encoding polypeptides with putative acetyl-CoA acetyltransferase activity were identified in the enterococci, although the genomes are incomplete. The other enzymes of the mevalonate pathway are less well studied, although several catalytic residues have been identified and are conserved in the enzymes of the gram-positive cocci (Fig. 2).

Our analysis suggests that gram-positive cocci and *B. burgdorferi* probably acquired all the IPP biosynthesis pathway genes through one or two horizontal gene transfer events. A primitive eukaryotic genome probably was the single source for the mevalonate pathway genes, since the archaeal species are missing identifiable *mvaK1* and *mvaD* genes and since the phosphomevalonate kinase of higher eukaryotes (11) shows no sequence homology to that from bacteria or *Saccharomyces cerevisiae* (51). Furthermore, with the exception of HMG-CoA reductase, all the phylogenies suggest that the bacterial enzymes are more closely related to those of eukaryotes than to those of the archaea. Horizontal gene transfers from eukaryotes to bacteria of genes essential for cell viability have been reported previously (9, 10). In nearly all instances, phylogenetic analyses suggest that gene transfers occurred very early in eukaryotic evolution, prior to the divergence of plants, animals, and fungi (8).

The consistent organization of mevalonate pathway genes in the gram-positive cocci is notable and may indicate a common mechanism for the regulation of the pathway; it might also provide clues to the mode of gene acquisition. *mvaK1*, *mvaD*, and *mvaK2* are organized in an identical operon in all species. *mvaS* and *mvaA* (or *mvaE*) are adjacent on the chromosome but are some distance from the other mevalonate pathway genes, except in *S. pyogenes*, where the genes are clustered. Gram-positive cocci probably acquired all of the genes involved in IPP biosynthesis via one or two horizontal transfer events. A possible scenario is suggested by the highly conserved genomic organization of the IPP biosynthetic genes. The ancestral gram-positive coccal species might have first acquired *mvaA* and initially utilized HMG-CoA reductase for mevalonate catabolism similar to *P. mevalonii*. Subsequently, *mvaS* and *mvaK1DK2* were acquired, permitting the biosynthesis of IPP via mevalonate. In *B. burgdorferi*, the clustering of mevalonate pathway genes is more tightly linked, suggesting that this organism participated in a separate genetic exchange with a eukaryotic cell. The tight linkage of mevalonate pathway genes in eubacteria further supports recent theories that genes in operons encoding single metabolic functions are highly favored for horizontal transfer between organisms since the recipient bacteria would acquire a novel metabolic function conferring a selective advantage (25, 26).

All five genes encoding enzymes of the mevalonate pathway were essential for the growth of *S. pneumoniae* in vitro under standard conditions and are envisaged to be essential in the other gram-positive cocci, since they also lack genes predicted to encode the enzymes of the GAP-pyruvate pathway. *S. pneumoniae* R6 *mvaS* null mutants grew as well as the wild type in the presence of mevalonate, although no *mvaA* null mutants could be obtained under similar conditions. Since both *mvaS*

FIG. 2. Alignment of the conserved regions of the mevalonate pathway enzymes from gram-positive bacteria, *B. burgdorferi*, *P. mevalonii*, and eukaryotes. Bold type indicates that amino acids are identical in all proteins. The asterisks indicate amino acids proposed to function in catalysis and substrate binding. Dashes indicate gaps introduced into the sequence to optimize the alignment. Numbers refer to amino acid positions. In all alignments, abbreviations are as follows: EFA, *E. faecalis*; EFM, *E. faecium*; SEP, *S. epidermidis*; SHA, *S. haemolyticus*; SAU, *S. aureus*; SPN, *S. pneumoniae*; SPY, *S. pyogenes*; BBO, *B. burgdorferi* (AE001169). In HMG-CoA synthase, abbreviations are as follows: SCA, *Staphylococcus carnosus* (U450157); HUM, human cytoplasmic enzyme (Q01581); MIT, human mitochondrial enzyme (NP005509); SAC, *S. cerevisiae* (P54839). In HMG-CoA reductase, abbreviations are as follows: PME, *P. mevalonii* (P13702). In mevalonate kinase, abbreviations are as follows: HUM, human (NP000422); SAC, *S. cerevisiae* (NP000422). In phosphomevalonate kinase, abbreviations are as follows: SAC, *S. cerevisiae* (P24521). In mevalonate diphosphate decarboxylase, abbreviations are as follows: HUM, human (AAC50440); SAC, *S. cerevisiae* (AAC49252).

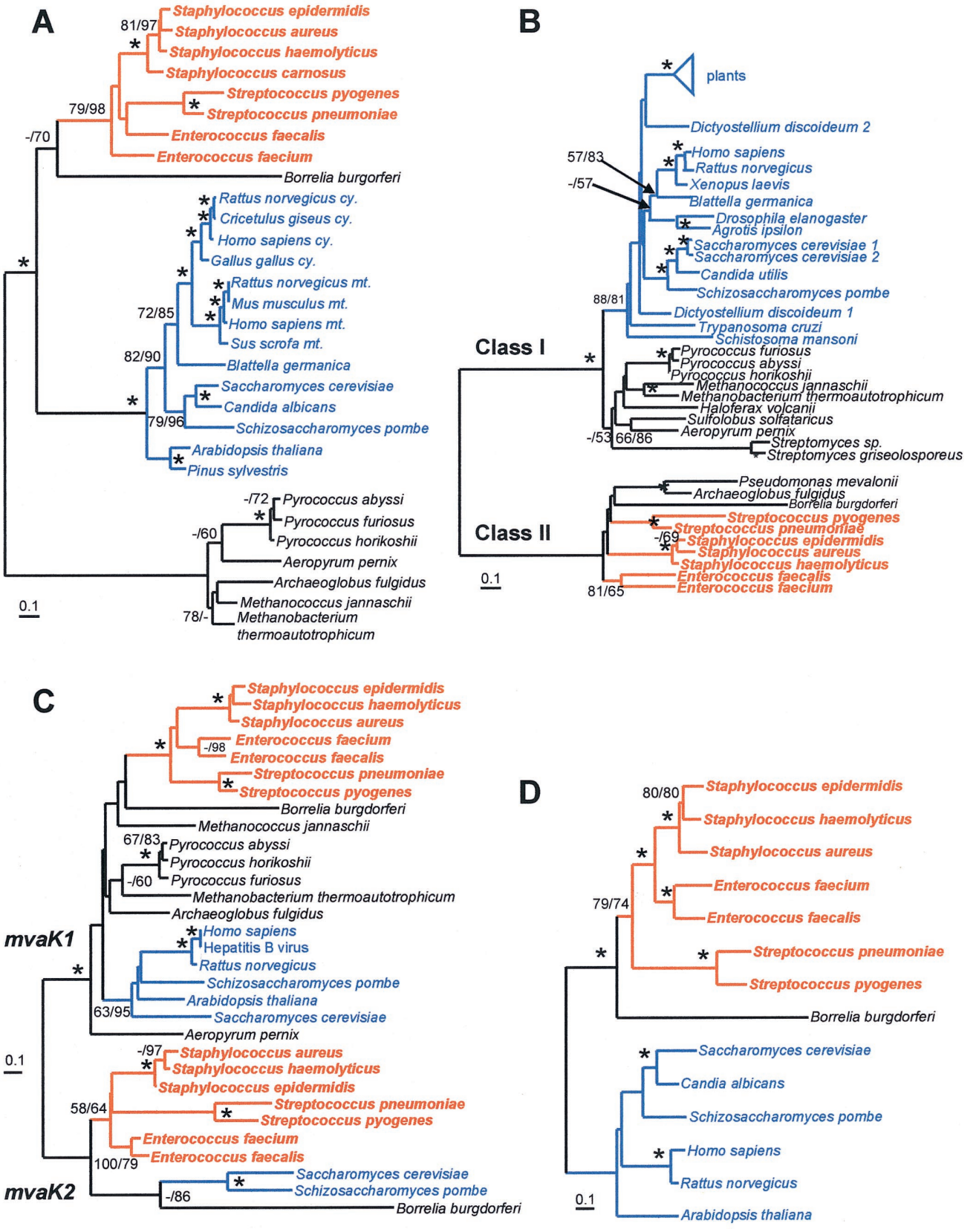

FIG. 3. Phylogenetic trees of HMG-CoA synthase (A), HMG-CoA reductase (B), mevalonate and phosphomevalonate kinase (C), and mevalonate diphosphate decarboxylase (D). Trees were constructed using the NJ method as implemented by the program NEIGHBOR of the PHYLIP 3.57c package (16). The scale bar represents 0.1 expected amino acid replacements per site as estimated by the program PROTDIST using the Dayhoff PAM-120 substitution matrix. Branches containing or linking low-G+C gram-positive coccal species are colored red, those of eukaryotes are blue, while those of archaea and other bacteria are black. Numbers at the branching points represent the percent occurrence in 1,000 random bootstrap replications of MP and NJ analyses. Values less than 50% are not shown or are indicated by a dash (–), while nodes supported in more than 90% of bootstrap replications for both methods are marked with an asterisk (*). Nodes separating classes I and II of HMG-CoA reductase as well as the mevalonate (*mvaK1*) and phosphomevalonate (*mvaK2*) kinases are indicated.

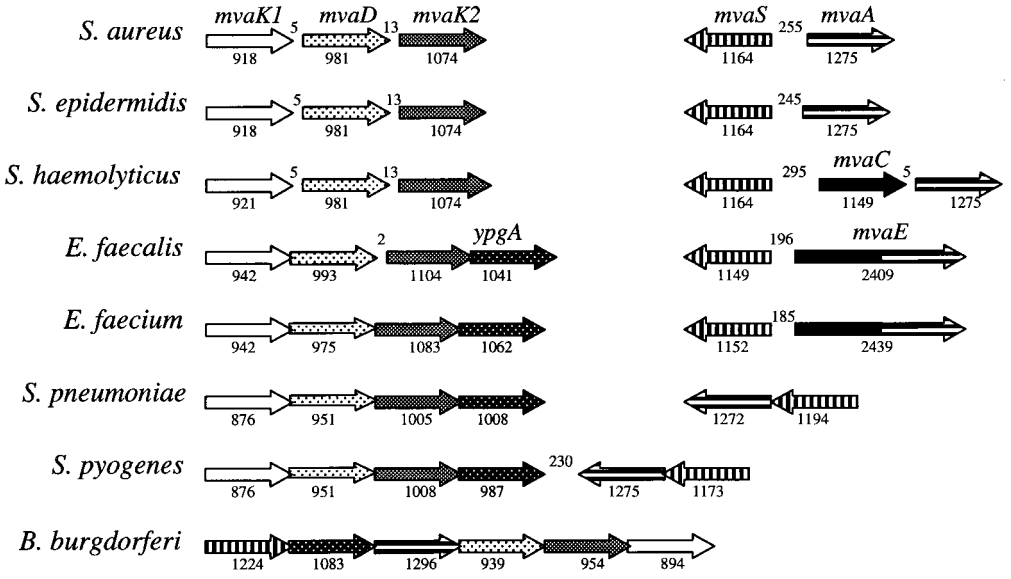

FIG. 4. Organization of the mevalonate pathway genes in gram-positive cocci and *B. burgdorferi*. Numbers above the genes indicate the number of nucleotides between genes, and numbers below indicate the length of each gene. *ypgA* has homology to a gene clustered with carotenoid biosynthetic genes in *E. herbicola* (22).

and *mvaA* could be replaced simultaneously in a double knockout, it is possible that deletion of *mvaA* alone led to a lethal intracellular accumulation of HMG-CoA. Removal of mevalonate from the medium prevented growth of the *mvaS* null mutant, although the mutant remained viable. The dependency of these allelic replacement mutants on mevalonate for growth supports the proposed functions of the genes. Inhibitors of HMG-CoA synthase are predicted to have a bacteriostatic

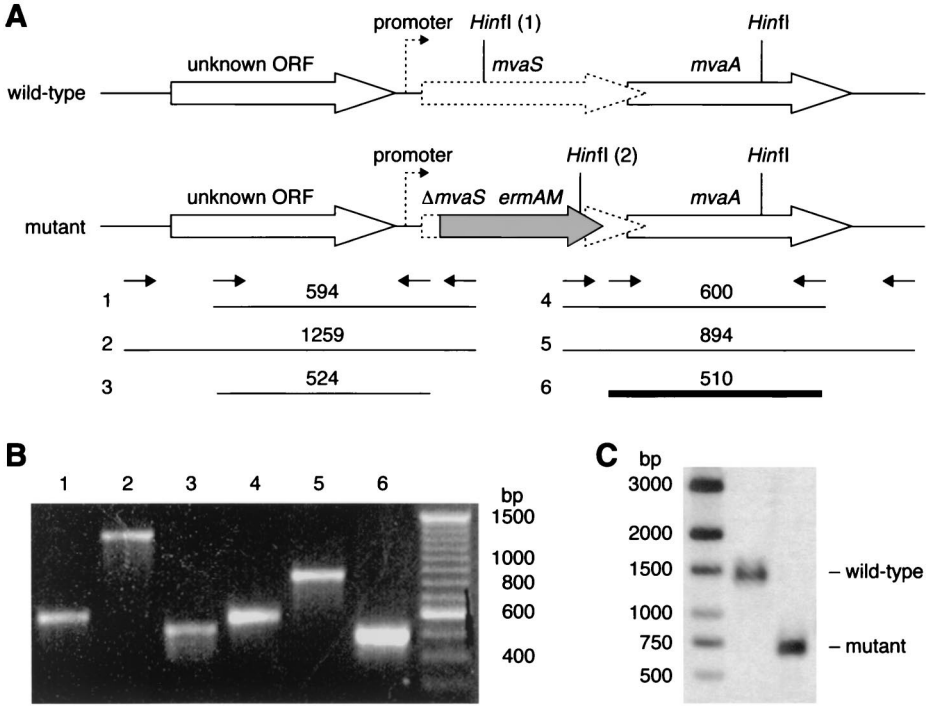

FIG. 5. Chromosomal analysis of the *S. pneumoniae mvaS* null mutant. (A) The chromosome of the *mvaS* null mutant contains an allelic replacement of *mvaS* (dotted block arrow, 1,194 bp) from nucleotides 166 to 1161 with the *ermAM* gene (gray block arrow). The translationally coupled downstream gene (*mvaA*) and the putative upstream promoter (dotted arrow) remain intact in the mutant. Oligonucleotide primers (small arrows) were designed to hybridize within the *ermAM* gene and to regions flanking *mvaS* and were used in PCRs against chromosomal DNA template prepared from the mutant. (B) Reactions 1, 2, 4, and 5 generated characteristic PCR products (shown), which could not be amplified with chromosomal DNA from the wild-type parent strain (not shown). Reactions 3 and 6 generated control fragments, which could be amplified in the mutant and parent strains. (C) Wild-type and mutant chromosomal DNA was restricted with *Hin*fI, subjected to agarose gel electrophoresis, and probed with labeled PCR fragment 6 (bold line). The predicted band sizes are 696 bp in the mutant and 1396 bp in the wild-type strain, since the left *Hin*fI site (labeled 1 in panel A) is deleted in the mutant and replaced by the *ermAM* gene containing another *Hin*fI site (labeled 2).

TABLE 1. Distribution of mevalonate and GAP-pyruvate pathways for IPP biosynthesis

Organism	Presence of:		Reference(s)
	Mevaolonate pathway	GAP-pyruvate pathway	
Gram-negative bacteria			
Enterobacteriaceae, *Haemophilus influenzae*	−	+	21, 35, 37
B. burgdorferi, *M. fulvus*, *C. ethylica*	+	−	21, 35
Gram-positive bacteria			
Mycobacteria, *B. subtilis*	−	+	35, 52
Streptomyces spp.[a]	+	+	41
Low-G+C gram-positive cocci	+	−	This study
Archaea	+	−	5, 6
Fungi	+	−	28
Higher plants			
Cytoplasmic	+	−	17
Plastid	−	+	29
Algae	+[b]	+[c]	14, 40, 43
Animals	+	−	17

[a] Some species of *Streptomyces* possess both pathways simultaneously.
[b] Including species such as *S. obliquus*, *Chlorella fusca*, and *Chlamydomonas reinhardtii*.
[c] Including species such as *E. gracilis*.

rather than a bacteriocidal effect, although inhibitors of HMG-CoA reductase would probably have a bacteriocidal effect on *S. pneumoniae*. The *mvaS* null mutant was severely attenuated in the murine respiratory tract infection model, and no bacteria were recovered at either the early or late stages of infection, suggesting that the bacteria are rapidly cleared within the first 12 h. The mevalonate content of plasma in humans and rats has been estimated to be 0.02 to 0.08 μM and 0.08 to 0.50 μM, respectively (34), both of which are well below the concentration of mevalonate required to support the growth of the *mvaS* null mutant on rich medium in vitro.

This study shows that, unlike *B. subtilis* and the gram-negative bacteria except *B. burgdorferi*, the gram-positive cocci possess the mevalonate pathway for IPP biosynthesis (Table 1). The essentiality of the mevalonate pathway for growth and the high sequence divergence from eukaryotic homologs identifies its components as potential antibacterial targets and exemplifies the use of comparative genomics to define novel targets for drug intervention against life-threatening, multidrug-resistant gram-positive cocci.

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