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Identification of 113 conserved essential genes using a high-throughput gene disruption system in *Streptococcus pneumoniae*

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ABSTRACT

The recent availability of bacterial genome sequence information permits the identification of conserved genes that are potential targets for novel antibiotic drug discovery. Using a coupled bioinformatic/experimental approach, a list of candidate conserved genes was generated using a Microbial Concordance bioinformatics tool followed by a targeted disruption campaign. Pneumococcal sequence data allowed for the design of precise PCR primers to clone the desired gene target fragments into the pEVP3 'suicide vector'. An insertion–duplication approach was employed that used the pEVP3 constructs and resulted in the introduction of a selectable chloramphenicol resistance marker into the chromosome. In the case of non-essential genes, cells can survive the disruption and form chloramphenicol-resistant colonies. A total of 347 candidate reading frames were subjected to disruption analysis, with 113 presumed to be essential due to lack of recovery of antibiotic-resistant colonies. In addition to essentiality determination, the same high-throughput methodology was used to overexpress gene products and to examine possible polarity effects for all essential genes.

INTRODUCTION

The publication of the genome of the first free-living organism, *Haemophilus influenzae*, (1) initiated the age of bacterial genomics (see http://www.tigr.org/tdb/mdb/mdbcomplete.html). The dozens of public and proprietary bacterial genomes have made available a wealth of new information and promise to greatly enhance our knowledge of a wide variety of different microbes. Along with this new genomic information, methodologies and tools have either been improved or developed *de novo* in order to analyze and apply the new data. Bioinformatics, the melding of biological sequence information, specific analysis software and high-speed computer tools, has become a crucial part of the genomic analysis process, both in assembling and interpreting vast amounts of sequence information. An important consequence of the microbial genomics era is that it will provide a number of essential, validated targets as possible candidates for the discovery of new antimicrobials. These validated drug targets will encode proteins either required for cell growth *in vitro* or for survival and/or virulence in the infected host. Presumably, screening against such novel targets for functional inhibitors will result in the discovery of novel therapeutic compounds active against bacteria, including the increasing number of antibiotic-resistant clinical strains that are the focus of much concern in clinical infectious disease.

An example of a bioinformatic approach to target identification was reported recently in a concordance analysis of microbial genomes (2). This system performs a FASTA comparison of multiple genomes at the amino acid level, building a relational database. The user queries this database and, by a web-based interface, retrieves related sequences with user-specified scores. This particular concordance demonstration compared the *Escherichia coli* genome against *Bacillus subtilis*, *H.influenzae*, *Helicobacter pylori* and *Mycobacterium tuberculosis* and subtracted out sequences with similarity greater than a selected exclusion criterion against the eucaryotic yeast *Saccharomyces cerevisiae.* This step was taken to reduce the potential cross-reaction of inhibitors with eucaryotic proteins. Genes in common with all five bacterial species were identified and a subset of sequences eliminated because of similarity to yeast sequences. The utility of such an approach was demonstrated by examination of the sequences selected, which included known drug targets. For example, the *gyrA* gene encoding DNA gyrase, the target of the quinolone class of antibiotics, was identified, as was *murA*,

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the target for fosfomycin. Several previously reported essential genes were also found, including *dnaA*, *ftsZ* and *mraY*. Different organisms and more or less stringent criteria can be used for such analyses depending on the desired end-point.

However, despite the best of bioinformatic approaches, it is necessary to validate the essentiality of antibacterial targets by experimental means, and several methods to accomplish this have been previously described (3–7). One approach made possible by the availability of genomic information is targeted gene disruption or 'gene knockouts'. One can attempt to specifically disrupt the desired gene in the chromosome using a selectable marker, such as drug resistance. If one can successfully recover the antibiotic resistance in a disrupted gene, this implies non-essentiality for that target, while lack of recovery of antibiotic-resistant colonies suggests essentiality. In this work, we describe a method to identify conserved bacteria-specific genes followed by high-throughput gene disruptions using the Gram-positive bacterium *Streptococcus pneumoniae* as a model system. Most of the steps in this process were successfully adapted to a 96-well microtiter plate format. Over 300 conserved candidate genes were disrupted in a relatively short time period, resulting in the identification of 113 conserved essential genes. The functions of the gene products encompassed a broad variety of cellular processes, including cell wall biosynthesis, DNA replication, cell division and fatty acid biosynthesis among others. In addition, a similar high-throughput approach was used for gene product expression and polarity analysis of the identified target genes.

MATERIALS AND METHODS

Bioinformatic analyses

Genome sequence data were obtained from a proprietary database (Pathogenome; Genome Therapeutics Corp., Waltham, MA) or obtained from the public domain (e.g. GenBank and TIGR). Genes were found using programs such as MAGPIE (8), loaded into the Concordance database, and desired genes selected using various parameters (2; see Table 1). Following experimental work, genes of interest were further analyzed with a variety of search methods. For global sequence similarity, conserved essential gene sequences were searched against a non-redundant protein database using the BLAST2 algorithm (9). In addition, similar genes in the Concordance database were identified using the 'Neighbors' function (2). To determine if the predicted gene sequences were full length and of proper reading frame, both BLAST searching and CLUSTAL multiple sequence alignments (10) were used along with rules for bacterial start site prediction (11). Putative gene function was assigned based on sequence similarities to genes with established function. Annotations were examined for potential transitive errors with MAGPIE and other informatics tools. For local sequence similarity, sequences were searched for Prosite (12) and Pfam (13) motifs. In addition to sequence similarity searching methods, other analyses were performed in an effort to try to determine the function of the conserved essential genes. Protein threading analyses were performed, using the ProCeryon fold recognition program (M.Sippl, unpublished results). Since genes of related function are often organized into operons in bacteria, operon structure in *S.pneumoniae* was examined and compared to that found in other bacteria (MAGPIE). Finally, metabolic pathway analysis was performed using Pathway Tools from DoubleTwist, which is based on the EcoCyc system (14).

Bacterial strains and media

Escherichia coli LE392 (15) was used as the strain for propagation of plasmid pEVP-3 (16). The strain was maintained on Luria (LB) agar (Gibco BRL, Life Technologies, Rockville, MD) and grown in liquid LB, at 37°C with shaking. *Streptococcus pneumoniae* strain Rx-1 (17) was maintained on blood agar plates at 37°C in 5% CO_2. The cells were grown in liquid culture in Todd-Hewitt (Difco, Becton-Dickinson, Sparks, MD) plus 0.5% yeast extract (Difco) at 37°C.

Purification of *S.pneumoniae* chromosomal DNA

Streptococcus pneumoniae Rx-1 was grown overnight on a blood agar plate at 37°C in 5% CO_2. Growth was removed with an inoculating loop and resuspended in 20 ml of Todd-Hewitt, 0.5% yeast extract with 400 μg/ml sterile sodium bicarbonate. The cells were grown at 37°C to an $OD_{600\ nm}$ of 0.4–0.6, chilled on ice, harvested by centrifugation at 5000 *g* for 15 min at 4°C, the pellet resuspended and washed once with 20 ml of ice-cold 10 mM Tris–HCl, pH 8.0, and 1 mM EDTA buffer, centrifuged as above and the resulting pellet quick frozen at –20°C. The cells were thawed and resuspended in 5 ml of the above Tris–HCl, EDTA buffer, and 0.005% sodium deoxycholate and 0.01% SDS added. After incubation at 37°C for 10 min, during which time the cells lysed, 500 μg/ml proteinase K (Sigma Chemical Co., St Louis, MO) was added and incubated for an additional 10 min. The cell lysate in buffer was gently extracted by inversion with an equal volume of phenol/chloroform/isoamyl alcohol (Gibco BRL). After centrifugation at 8000 *g* for 10 min, the upper aqueous layer was removed and extracted twice with an equal volume of chloroform/isoamyl alcohol. The final aqueous extract was made up to 0.3 M sodium acetate, and 2.2 vol of ethanol were overlayed. The DNA was spooled onto a glass rod and redissolved in 2 ml of 10 mM Tris–HCl, pH 8.0, 1 mM EDTA overnight at 4°C. This preparation was dialyzed against 400 vol of the same Tris–HCl, EDTA buffer before storage at 4°C. DNA concentration was determined by absorbance at 260 nm and adjusted to 0.5 μg/μl.

Primer construction

Knockout (K/O) primers were designed from sequences of annotated reading frames and were usually ~25 bp in length, ~50% GC content and located at least 100 bp internal to the N- and C-termini of the predicted full-length target gene, and generated 300–600 bp fragments. A list of primers used in this work is available as Supplementary Material. Primer design and ordering (Life Technologies) were done electronically and primers were received arrayed in 96-well plate format when appropriate.

Genomic PCR fragment generation

PCRs were run in a 96-well plate format using 50 μl total reaction volumes that contained the following: 36 μl dH_2O, 5 μl 10× Vent buffer (New England Biolabs, Beverly, MA), 1 μl K/O forward primer (0.5 μg/μl), 1 μl K/O reverse primer (0.5 μg/μl), 0.5 μl Vent DNA polymerase (2000 U/ml) (New

England Biolabs), 1.5 µl each (6.0 µl total) dNTPs (10 mM) (New England Biolabs) and 0.5 µl *S.pneumoniae* chromosomal DNA (0.5 µg/µl). The PCR program was as follows: 95°C for 5 min, then 30 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 30 s, followed by 72°C for 10 min and a 4°C hold. PCR Purification Kits, 96-well format and individual columns (Qiagen, Valencia, CA), were used to purify PCR products. Products (5 µl each) were visualized by a 96-well agarose gel electrophoresis system (Life Technologies).

Ligation reactions

Blunt-end ligation reactions were set up in a 96-well plate format as follows: 10 µl genomic PCR fragment (~100–500 ng), 1.0 µl pEVP3 *Sma*I-digested vector (~10 ng), 1.5 µl 10× ligation buffer (New England Biolabs), 1.0 µl T4 DNA ligase (400 000 U/ml) (New England Biolabs) and 1.5 µl dH_2O for 15 µl total reaction volumes. Reactions were incubated at 14°C overnight.

Escherichia coli transformations

Bacterial transformations with the ligated pEVP3 plasmid were set up in a 96-well plate using a 3 µl ligation reaction and 50 µl *E.coli* LE392 competent cells (*E.coli* Genetic Stock Center, Yale University, New Haven, CT). Plates were incubated on ice for 30 min, followed by heat shock at 42°C for 90 s, and returned on ice for 2 min. Aliquots of 100 µl SOC medium (Life Technologies) were added to the cells and plates were incubated at 37°C on a platform shaker for 1 h. Each transformation reaction (total volume) was plated on LB/chloramphenicol (15 µg/ml) agar in six-well plate format and grown overnight at 37°C to allow for colony formation.

Colony PCR

Screening for the recombinant clones that contained inserts was done by colony PCR in 96-well plate format. Transformed *E.coli* colonies were picked by sterile pipet tip and resuspended in 50 µl dH_2O. These colony suspensions were stored at 4°C in 96-well plates and subsequent positive clones of interest were plated onto solid medium and saved. PCRs included the following: 36.5 µl dH_2O, 0.5 µl pEPV3 forward primer (0.25 µg/µl), 0.5 µl pEPV3 reverse primer (0.25 µg/µl), 1.5 µl each (6.0 µl total) dNTP (10 mM), 0.5 µl Vent DNA polymerase, 5 µl 10× Vent buffer and 1 µl of a 1:50 cell dilution for a 50 µl total reaction volume. The pEPV3 cloning site flanking forward primer was 5′-CATCAAGCTTATCGATACCGTCG-3′ and the reverse flanking primer 5′-CACAGTAGTTCACCACCTTTTCCC-3′ (Life Technologies). The PCR program was 95°C for 5 min, followed by 30 cycles of 95°C for 1 min, 58°C for 1 min and 72°C for 30 s, then 72°C for 10 min with a 4°C hold. PCR products (10 µl each) were visualized by 96-well agarose gel electrophoresis after staining with ethidium bromide. Constructs that were negative for a targeting DNA fragment insert produced a 100 bp control fragment. Those that were positive for an insert produced an ~400–700 bp fragment, depending on the targeting insert size. Miniprep cultures of positive clones were inoculated directly from cell dilution plates.

Miniprep and clone confirmation

Positive constructs were inoculated into 5 ml LB/chloramphenicol medium and incubated overnight at 37°C with aeration. Miniprep DNA was prepared by standard procedure using the Qiagen 96-well Turbo Prep System or individual columns. PCR with ORF-specific primers was used to confirm the presence of the correct insert. Reactions in 96-well plates included: 35.5 µl dH_2O, 5 µl 10× Vent buffer, 1 µl K/O forward primer (0.5 µg/µl), 1 µl K/O reverse primer (0.5 µg/µl), 0.5 µl Vent DNA polymerase (2000 U/ml), 1.5 µl each (6.0 µl total) dNTPs (10 mM) and 1.0 µl miniprep DNA for a 50 µl total reaction volume. The PCR program and agarose gel analysis of products was as described above.

Streptococcus pneumoniae competent cell preparation and gene disruption

One colony, from a fresh blood agar plate, was resuspended in 1.5 ml Todd-Hewitt medium (Difco) plus 0.5% yeast extract (Difco). An aliquot of 100 µl of this dilution was used to inoculate 50 ml of the same medium and grown overnight at 37°C. The next morning, 5 ml of the overnight culture was added to 45 ml fresh medium and grown at 37°C to an OD_{600} of 0.25 (~4–5 h). Sterile glycerol to a final concentration of 10% was added, cells were aliquoted in 1 ml volumes, frozen in a dry ice–ethanol bath and stored at –80°C.

Pneumococcal transformations were set up in 96-well plate format and included the following: 1 µg miniprep pEVP3 plus insert DNA (~10 µl Qiagen miniprep DNA) plus 200 µl *S.pneumoniae* Rx-1 competent cells diluted 1:10 in competence medium (18,19). Competence medium contained Todd-Hewitt broth plus 0.5% yeast extract, 0.2% BSA, 0.01% $CaCl_2$ and 100 ng/ml peptide pheromone Csp-1 (H-EMRLSKFFRDFILQRKK-OH) (ResGen, Huntsville, AL). A non-essential (pEPV3::*lytA*) and essential (pEPV3::*ftsZ*) disruption control construct was run with each experiment. Cells were incubated at 37°C for 2.5–3 h without shaking. All 200 µl were plated on Todd-Hewitt agar with 0.5% yeast extract and 2 µg/ml chloramphenicol and incubated for 24 h at 37°C in a CO_2 incubator. Plates were then examined and colony numbers compared to controls. The *lyt*A non-essential controls typically gave 150–200 colonies per transformation and the *fts*Z positive controls gave 0 colonies per transformation. Experimentally, most non-essential genes gave 200–300 colonies per transformation (*lyt*A was found to be on the low end of the spectrum) and essential genes gave results similar to *fts*Z. An occasional single chloramphenicol-resistant colony was seen for some experiments, presumably due to contamination or non-homologous integration of the construct. For verification, all essential genes were subjected to a second independent gene disruption experiment.

Polarity analysis of essential ORFs

Primers for the N- and C-termini were designed from curated sequence data of full-length genes. Appropriate restriction enzyme sites were added to primers for cloning and subsequent expression of protein in the *E.coli* BL21 pET vector system (pET21b and pET21+; Novagen). PCR of full-length genes from genomic DNA was as described above. Full-length genes were cloned as blunt fragments into pEPV3 for polarity

Table 1. Sample concordance

Target organism	*Streptococcus pneumoniae*
Number of sequences	2345
Compared against	*Bacillus subtilis*
	Enterococcus faecalis
	Escherichia coli
	Staphylococcus aureus
Selection qualifier	Target sequences must match at least two species
Selection criterion	Overlap ratio >0.4[a]
Number of sequences which match at least one species	746
Sequences removed by selection requirement	265
Excluding organism(s)	Yeast (YPD)
Exclusion criterion	Overlap ratio >0.3[b]
Sequences removed by exclusion	83
Number of sequences in concordance	398

[a]≥40% amino acid sequence identity.
[b]≥30% amino acid sequence identity.

testing in *S.pneumoniae*, similarly to the procedure described above for gene disruptions.

Expression of essential gene products

In a separate reaction, fragments were also digested with appropriate restriction enzymes and ligated with a similarly digested pET expression vector. High-throughput procedures for ligation and transformation were similar to those described previously. Ligations with pET vectors were transformed into *E.coli* DH5α for the identification of positive clones and then into appropriate strain backgrounds (*E.coli* BL21 [λDE3]) for protein expression according to the manufacturer's recommendations. A small-scale (5 ml) induction experiment was run on all new expression constructs and analyzed by SDS–PAGE for protein overexpression and correct size of the expressed protein.

DNA sequence analysis

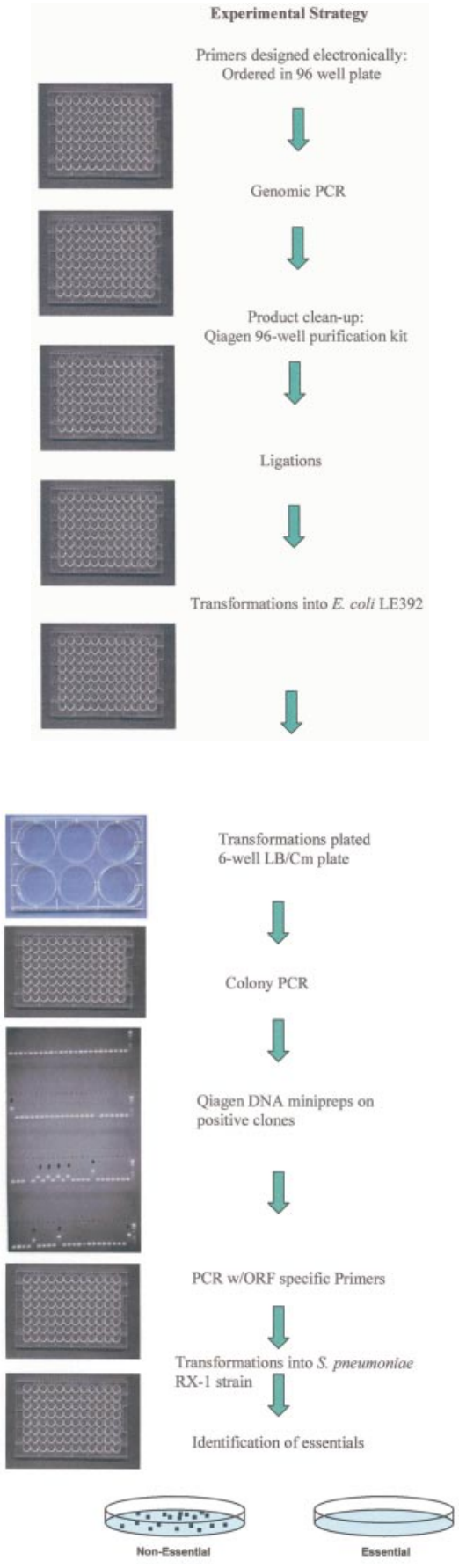
All expression constructs underwent DNA sequence analysis to confirm insert sequences. Constructs were sequenced using vector-specific primers, followed by primer walking with target gene-specific primers. The sequences were analyzed for proper reading frame and compared to other available sequence data.

RESULTS

In order to select potential target genes for essentiality analyses, the recently described Microbial Concordance tool (2) was employed. Entire genomes can be compared against other genomes of choice to obtain a list of genes common among several pathogens with the desired percent identity. In a similar manner, genes that are more specific to bacteria can be selected for by filtering out eucaryotic genes above a predetermined percent identity. Several different concordance analyses were run and the results were combined in order to obtain a list of candidate bacterial genes with potential broad spectrum. The concordance also screened genes for similarity against possible eucaryotic homologs, with the aim of minimizing potential adverse effects for a novel inhibitor by undesirable cross-reactivity. These genes were then selected as candidates for the high-throughput disruption experiments to determine essentiality. An example of a more recent concordance is illustrated in Table 1. The target genome, *S.pneumoniae*, was compared against the *B.subtilis*, *Enterococcus faecalis*, *E.coli* and *Staphylococcus aureus* (20) genomes. The selection criterion was 40% global amino acid sequence identity with matches in at least two bacterial species. *Streptococcus pneumoniae* sequences that were >30% identical to yeast proteins were excluded to reduce the possibility of target cross-reactivity. The result of this particular concordance analysis was a list of 398 candidate genes. Following several rounds of concordance analyses, a set of 347 candidate open reading frames was selected for subsequent targeted disruptions to establish essentiality.

The target genes were next analyzed to verify probable start and stop codons for the open reading frames. Subsequently, primers were designed from the annotated reading frame sequences and those were selected that were at least 100 bp internal to the start and stop codons of each gene. These 300–600 bp fragments were inserted into the pEVP3 vector (21) and initially transformed into *E.coli*. Selection for chloramphenicol-resistant transformants and screening in a 96-well format agarose gel allowed ready identification of pEVP3 plasmids with inserts of pneumococcal DNA (Fig. 1). The pEVP3 vectors that had inserts were subsequently transformed into *S.pneumoniae*. The cloned gene fragment promotes single crossover homologous recombination that, in a manner resembling a Campbell-like insertion (22), introduces via insertion–duplication the pEVP3 vector into the chromosome, effecting the disruption (Fig. 2). A cell in which a non-essential gene has been disrupted will survive the disruption and upon incubation form a macroscopic colony on a chloramphenicol-containing agar plate, by virtue of the chloramphenicol resistance encoded by the pEVP3 recombinant plasmid integrated into the chromosome. A disrupted essential gene, on the other hand, will not permit colony formation and thus no chloramphenicol-resistant colonies will be recovered. Table 2 lists the resulting 113 genes derived from the above essentiality testing. Table 3 lists the 234 genes disrupted that were non-essential. Table 4 catagorizes the essential genes based on available annotations.

Similar high-throughput approaches were used to overexpress *S.pneumoniae* gene products of interest in *E.coli* and to determine whether there were potential polarity issues for these candidate genes (Fig. 3). Since many bacterial genes are co-transcribed from a common upstream promoter, gene disruptions can cause downstream polar effects that can



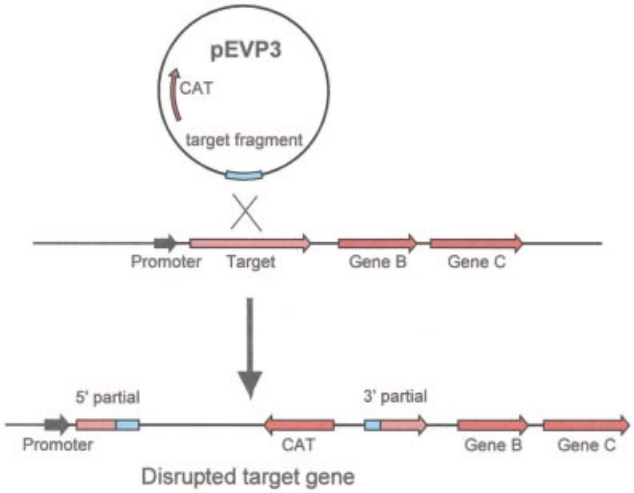


Figure 2. Use of plasmid pEVP3 for disruption of pneumococcal genes. An internal gene fragment was cloned into this vector which is incapable of replication in pneumococci. Selection for the insertion–duplication event was on medium containing chloramphenicol.

mislead in the determination of essentiality. By using a full-length target gene sequence cloned into the pEVP3 vector, the insertion–duplication event results in the duplication of the entire target gene (Fig. 4). If chloramphenicol-resistant colonies are now obtained, the assumption is made that the gene product is indeed essential to the bacterium. If no colonies are detected, the assumption is that one or more downstream transciptionally linked genes in an operon are essential for growth (Table 2, +). In the latter case, additional experimental data are required to establish which gene or genes are essential in the putative operon. Genes that display no polarity effects (Table 2, –) can be further advanced as targets for novel antibacterials.

For protein overexpression, the full-length target gene sequence was cloned into pET vectors, transformed into *E.coli* for verification of the construct and transformed into an expression strain of *E.coli.* Following protein expression and analysis by SDS–PAGE, proteins can be further purified either with Ni–NTA columns for those proteins with a C-terminal 6-histidine tag or via conventional purification strategies for untagged proteins.

DISCUSSION

The recent availability of bacterial genomic sequence information has, among many uses, allowed the identification of potential novel, conserved antibacterial targets (23,24). These novel targets could be the basis for the identification of fundamentally new classes of antibiotics. The need for novel antimicrobials has been emphasized by the continued increase in the number of bacterial pathogens that possess multiple

Figure 1. Experimental strategy for high-throughput disruption of pneumococcal target genes. Steps performed in 96-well microtiter plates are indicated. *Escherichia coli* transformations were plated on six-well plates while *S.pneumoniae* transformations were plated on standard size 15 × 100 mm plates.

Table 2. Conserved essential bacterial genes

K/O Number	TIGR SP Gene	Annotation	Polarity[a]
1	SP0004	GTP-binding protein	+
2	SP0005	peptidyl-tRNA hydrolase	-
3	SP0012	hypoxanthine-guanine phosphoribosyltransferase	-
4	SP0121	metallo-beta-lactamase superfamily protein	-
5	SP0122	conserved hypothetical protein	+
6	SP0129	glycoprotease family protein	-
7	SP0179	Holliday junction DNA helicase RuvA	-
8	SP0192	conserved hypothetical protein	+
9	SP0193	conserved hypothetical protein, authentic frameshift	-
10	SP0259	Holliday junction DNA helicase RuvB	-
11	SP0263	eep protein	+
12	SP0266	glucosamine--fructose-6-phosphate aminotransferase	+
13	SP0273	translation elongation factor G	+
14	SP0334	ylIC protein	+
15	SP0337	phospho-N-acetylmuramoyl-pentapeptide-transferase	+
16	SP0347	capsular polysaccharide biosynthesis protein Cps4B	n.d[b]
17	SP0370	recombination protein U	+
18	SP0402	signal peptidase I	-
19	SP0419	enoyl-(acyl-carrier-protein) reductase	+
20	SP0420	malonyl CoA-acyl carrier protein transacylase	+
21	SP0421	3-oxoacyl-[acyl-carrier protein] reductase	+
22	SP0422	3-oxoacyl-(acyl-carrier-protein) synthase II	+
23	SP0423	acetyl-CoA carboxylase, biotin carboxyl carrier protein	+
24	SP0424	(3R)-hydroxymyristoyl-(acyl-carrier-protein) dehydratase	+
25	SP0425	acetyl-CoA carboxylase, biotin carboxylase	+
26	SP0426	acetyl-CoA carboxylase, carboxyl transferase, beta subunit	+
27	SP0427	acetyl-CoA carboxylase, carboxyl transferase, alpha subunit	-
28	SP0433	N utilization substance protein B	-
29	SP0435	translation elongation factor P	+
30	SP0457	bacitracin resistance protein	-
31	SP0499	phosphoglycerate kinase	+
32	SP0554	conserved hypothetical protein	+
33	SP0580	acetyltransferase, GNAT family	+
34	SP0581	phenylalanyl-tRNA synthetase, beta subunit	-
35	SP0613	metallo-beta-lactamase superfamily protein	-
36	SP0672	GTP-binding protein HflX	-
37	SP0674	metallo-beta-lactamase superfamily protein	-
38	SP0741	conserved hypothetical protein	-
39	SP0755	peptide chain release factor 2, authentic frameshift	n.d[b]
40	SP0778	16S rRNA processing protein RimM	+
41	SP0779	tRNA (guanine-N1)-methyltransferase	-
42	SP0803	rod shape-determining protein RodA, putative	-
43	SP0806	DNA gyrase subunit B	+
44	SP0807	septation ring formation regulator EzrA, putative	-
45	SP0839	pantothenate kinase	-
46	SP0864	conserved hypothetical protein	+
47	SP0865	DNA polymerase III, gamma and tau subunits	+
48	SP0873	membrane protein	-
49	SP0895	DNA polymerase III, alpha subunit	n.d.[b]
50	SP0913	ABC transporter, permease protein, putative	n.d.[b]
51	SP0936	DNA polymerase III, delta prime subunit	-
52	SP0945	ribosome recycling factor	-
53	SP0967	conserved hypothetical protein TIGR00043	+
54	SP0971	kinase, putative	-
55	SP0974	preprotein translocase, SecG subunit, putative	-
56	SP0988	UDP-N-acetylglucosamine pyrophosphorylase	-
57	SP1020	peptide chain release factor 1	-

antibiotic resistance (25,26). The combination of bioinformatic and laboratory approaches described here permits the assignment of potential targets conserved among pathogens as either essential or non-essential for *in vitro* survival on growth media. The previously described Microbial Concordance informatics tool (2) was used to first identify conserved open reading frames based on the assumption that these would have the best chance of yielding a broad spectrum inhibitor of bacterial growth. An entire genome can be compared against multiple genomes of choice to obtain a list of genes common among several pathogens, with the desired percent identity specified by the user. In a similar manner, the concordance permits the identification or subtraction of undesired gene sets, such as bacterial genes with a relatively high similarity to

Table 2. *Continued*

58	SP1067	cell division protein FtsW, putative	-
59	SP1079	GTP-binding protein, GTP1/Obg family	-
60	SP1081	UDP-N-acetylglucosamine 1-carboxyvinyltransferase	-
61	SP1097	conserved hypothetical protein	+
62	SP1098	conserved hypothetical protein	-
63	SP1113	DNA-binding protein HU	-
64	SP1155	GTP-binding protein	-
65	SP1227	DNA-binding response regulator	+
66	SP1244	signal recognition particle-docking protein FtsY	-
67	SP1247	conserved hypothetical protein	-
68	SP1263	DNA topoisomerase I	-
69	SP1270	alcohol dehydrogenase, zinc-containing	+
70	SP1271	cytidine diphosphocholine pyrophosphorylase, putative	+
71	SP1287	signal recognition particle protein	-
72	SP1288	conserved hypothetical protein	+
73	SP1421	conserved hypothetical protein	+
74	SP1517	transcription elongation factor GreA	-
75	SP1529	polysaccharide biosynthesis protein, putative	-
76	SP1530	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase	-
77	SP1534	inorganic pyrophosphatase, manganese-dependent	-
78	SP1540	single-strand binding protein	+
79	SP1542	asparaginyl-tRNA synthetase	-
80	SP1545	conserved hypothetical protein	+
81	SP1555	dihydrodipicolinate reductase	+
82	SP1559	phosphoglucomutase/phosphomannomutase family protein	-
83	SP1568	GTP-binding protein	-
84	SP1589	Mur ligase family protein	+
85	SP1590	conserved hypothetical protein	-
86	SP1598	phosphomethylpyrimidine kinase, putative	-
87	SP1610	Bcl-2 family protein	-
88	SP1616	ribulose-phosphate 3-epimerase family protein	n.d.[b]
89	SP1624	acyltransferase family protein	+
90	SP1645	GTP pyrophosphokinase	n.d.[b]
91	SP1648	manganese ABC transporter, ATP-binding protein	+
92	SP1661	cell division protein DivIVA	-
93	SP1665	ylmE protein	+
94	SP1726	3-hydroxy-3-methylglutaryl-CoA reductase	-
95	SP1732	serine/threonine protein kinase	-
96	SP1733	phosphatase, putative	+
97	SP1737	DNA-directed RNA polymerase, omega subunit, putative	+
98	SP1747	conserved hypothetical protein	-
99	SP1748	conserved hypothetical protein	+
100	SP1749	GTP-binding protein	-
101	SP1750	conserved hypothetical protein	+
102	SP1837	capsular polysaccharide biosynthesis protein, putative	-
103	SP1838	glycosyl transferase, putative	+
104	SP1887	oligopeptide ABC transporter, ATP-binding protein AmiF	-
105	SP1889	oligopeptide ABC transporter, permease protein AmiD	+
106	SP1906	chaperonin, 60 kDa	+
107	SP1968	phosphopantetheine adenylyltransferase	-
108	SP2040	jag protein, putative	-
109	SP2041	SpoIIIJ family protein	+
110	SP2203	replicative DNA helicase	+
111	SP2220	ABC transporter, ATP-binding protein	-
112	SP2228	inosine-5'-monophosphate dehydrogenase	n.d.[b]
113	no SP gene[c]	capsular polysaccharide biosynthesis protein CpsO	+

[a]–, polarity not an issue, see Results; +, polarity is an issue, see Results.
[b]n.d., not determined.
[c]Gene not present in the TIGR serotype 4 strain.

eucaryotic genes. Thus, open reading frames with high sequence identity to eucaryotic gene sequences can be reduced in priority as targets to enhance chances for novel inhibitor selectivity against bacteria. This was done initially with yeast sequence and later with human genomic sequence as it became available. Several concordance iterations and selection criteria were used in this work and results were pooled to yield the final list of 347 genes for target disruption analyses. One advantage of this approach is the flexibility for the user to set desired limits both for sequence identity and sequence exclusion. Since there are no optimal predetermined numbers for both, multiple analyses can be conducted at the bioinformatic level followed by experimental validation. Essential targets can be subsequently rank ordered with regard to their

Table 3. List of *S.pneumoniae* non-essential genes by gene disruption analysis[a]

K/O Number	TIGR SP Gene	Annotation
NSP-1	SP0006	transcription-repair coupling factor (mfd)
NSP-2	SP0013	cell division protein FtsH
NSP-3	SP0023	DNA repair protein RadA, authentic point mutation
NSP-4	SP0049	vanz protein; putative
NSP-6	SP0092	bacterial extracellular solute-binding protein
NSP-8	SP0104	hydrolase; haloacid dehalogenase-like family
NSP-9	SP0120	glucose-inhibited division protein A (gidA)
NSP-11	SP0204	acetyltransferase; GNAT family
NSP-12	SP0205	anaerobic ribonucleotide reductase activator protein
NSP-15	SP0278	aminopeptidase PepS (pepS)
NSP-17	SP0282	PTS system; mannose-specific IID component
NSP-19	SP0314	hyaluronidase
NSP-20	SP0318	carbohydrate kinase; PfkB family
NSP-21	SP0320	oxidoreductase; short chain dehydrogenase/reductase family
NSP-23	SP0330	RegR
NSP-24	SP0340	autoinducer-2 production protein (luxS)
NSP-26	SP0348	capsular polysaccharide biosynthesis protein Cps4C (cps4C)
NSP-28	SP0387	response regulator
NSP-29	SP0400	trigger factor (tig)
NSP-30	SP0403	ribonuclease hii (ec 3.1.26.4) (rnase hii) (rnhC)
NSP-31	SP0406	MutS2 family protein
NSP-32	SP0408	sodium:alanine symporter family protein
NSP-33	SP0439	peptide chain release factor 3 (prfC)
NSP-34	SP0458	DNA-damage inducible protein P (dinP)
NSP-37	SP0489	histidine kinase; putative
NSP-38	SP0496	Na/Pi cotransporter II-related protein
NSP-40	SP0557	ribosome-binding factor A (rbfA)
NSP-42	SP0601	transmembrane protein Vexp3 (vex3)
NSP-43	SP0603	response regulator
NSP-44	SP0611	single-stranded-DNA-specific exonuclease RecJ (recJ)
NSP-45	SP0615	beta-lactam resistance factor fibA
NSP-46	SP0628	HIT family protein
NSP-48	SP0675	oxidoreductase; short chain dehydrogenase/reductase family
NSP-49	SP0680	ribosomal small subunit pseudouridine synthase A (rsuA)
NSP-50	SP0681	elongation factor Tu family protein
NSP-51	SP0718	thiamin-phosphate pyrophosphorylase (thiE)
NSP-53	SP0724	hydroxyethylthiazole kinase; putative
NSP-54	SP0726	phosphomethylpyrimidine kinase (thiD)
NSP-55	SP0737	sodium-dependent transporter
NSP-56	SP0740	MutT/nudix family protein
NSP-58	SP0780	ribonucleoside-triphosphate reductase-related protein
NSP-60	SP0791	oxidoreductase; aldo/keto reductase family
NSP-61	SP0793	oxidoreductase; short chain dehydrogenase/reductase family
NSP-63	SP0817	MutT/nudix family
NSP-64	SP0835	purine nucleoside phosphorylase (deoD)
NSP-65	SP0837	DNA topology modulation protein FlaR; putative
NSP-66	SP0845	lipoprotein
NSP-67	SP0846	sugar ABC transporter, ATP-binding protein
NSP-68	SP0848	sugar ABC transporter; permease protein; putative
NSP-69	SP0878	stage III sporulation protein e
NSP-70	SP0881	thiazole biosynthesis protein ThiI (thiI)
NSP-71	SP0890	integrase/recombinase, phage integrase family
NSP-72	SP0908	regulatory protein Tex
NSP-73	SP0922	carbon-nitrogen hydrolase family protein
NSP-74	SP0923	Cof family protein
NSP-75	SP0928	lipoprotein signal peptidase (lspA)
NSP-76	SP0929	ribosomal large subunit pseudouridine synthase D (rluD)
NSP-77	SP0931	glutamate 5-kinase (proB)
NSP-79	SP0938	tetrapyrrole methylase family protein
NSP-82	SP0953	acetyltransferase; GNAT family
NSP-83	SP0955	competence protein CelB
NSP-84	SP0957	ABC transporter; ATP-binding protein
NSP-85	SP0969	GTPase Era (era)
NSP-86	SP0972	multi-drug resistance efflux pump
NSP-87	SP0980	O-methyltransferase
NSP-88	SP0989	MutT/nudix family protein
NSP-89	SP1014	dihydrodipicolinate synthase (dapA)
NSP-91	SP1029	RNA methyltransferase; TrmA family
NSP-93	SP1087	ATP-dependent DNA helicase PcrA (pcrA)
NSP-95	SP1099	ribosomal large subunit pseudouridine synthase; RluD subfamily
NSP-97	SP1110	macrolide-efflux protein
NSP-99	SP1118	pullulanase; putative
NSP-100	SP1123	glycogen biosynthesis protein GlgD (glgD)
NSP-102	SP1156	ribonuclease HII (rnhB)
NSP-103	SP1157	voltage-gated chloride channel family protein
NSP-105	SP1166	MATE efflux family protein
NSP-106	SP1171	hydrolase; haloacid dehalogenase-like family
NSP-107	SP1182	lactose phosphotransferase system repressor (lacR)
NSP-108	SP1186	PTS system; lactose-specific IIa component (lacF)
NSP-109	SP1187	transcription antiterminator LacT (lacT)
NSP-110	SP1197	PTS system; galactitol-specific IIB component
NSP-111	SP1201	serine/threonine protein phosphatase
NSP-112	SP1202	DNA repair protein RecN (recN)
NSP-113	SP1204	hemolysin A; putative
NSP-114	SP1205	geranyltranstransferase (ispA)
NSP-115	SP1215	transporter; fnt family
NSP-116	SP1225	vicX protein (vicX)
NSP-117	SP1235	MutT/nudix family protein
NSP-118	SP1238	excinuclease ABC; subunit B (uvrB)
NSP-119	SP1241	amino acid ABC transporter; periplasmic solute-binding protein
NSP-120	SP1245	Cof family protein
NSP-122	SP1266	DNA processing chain A (dprA)
NSP-123	SP1285	glucose-inhibited division protein B (gidB)

degree of amino acid similarity among pathogens and their distance from eucaryotic sequences.

Because of the availability of pneumococcal gene sequence information (27,28), a targeted as opposed to random gene disruption approach (29) was selected. *Streptococcus pneumoniae* strain Rx-1 (17) was chosen for this work, recognizing that there are DNA sequence polymorphisms among pneumococcal strains and that the use of other strains or essentiality testing methods may yield a few differences in results. Certain key advancements in the biology of *S.pneumoniae* have made this organism a premier model system for rapid gene knockouts. The identification of a small peptide regulator that induces competence for genetic transformation (18,19) has made manipulation of the system extremely reproducible and convenient. The *E.coli* plasmid pEVP3, which has a number of key features, including chloramphenicol resistance expression in both Gram-positive and Gram-negative bacterial backgrounds, was designed specifically by Morrison *et al.* (16) for genetic disruptions in pneumococci. To construct such gene disruptions, the internal gene fragments were cloned into pEVP3 with the recombinant plasmids inserting into the pneumococcal chromosome via homologous recombination into intact genes leading to targeted insertion–partial gene duplication events. The position of the gene disruption event (at least 100 bp from the gene start and stop sites) should be sufficient to ensure that the resulting insertion–duplication event does not restore gene function. It should be noted that our independently derived disruption criteria are similar to those used in another global mutagenesis campaign (30). Clearly an absolute requirement for this process to succeed was the availability of the DNA sequence of the pneumococcal target genes with correctly annotated start and stop signals. This allowed for the synthesis of the gene-specific primers needed for PCR generation of internal gene targeting fragments. We estimate that it would require 6 days for one person to take one plate (96 individual gene knockouts) and

Table 3. *Continued*

NSP-125	SP1291	Cof family protein
NSP-126	SP1369	prephenate dehydratase (pheA)
NSP-127	SP1370	shikimate kinase (aroK)
NSP-128	SP1376	shikimate dehydrogenase (aroE)
NSP-129	SP1377	3-dehydroquinate dehydratase (aroD)
NSP-131	SP1381	ABC transporter; ATP-binding protein
NSP-132	SP1396	phosphate ABC transporter; ATP-binding protein; putative
NSP-133	SP1403	inositol monophosphatase; putative
NSP-134	SP1416	S-adenosylmethionine tRNA ribosyltransferase-isomerase (queA)
NSP-135	SP1429	peptidase; U32 family
NSP-136	SP1434	NrpA; putative
NSP-137	SP1435	ABC transporter ATP-binding protein
NSP-140	SP1446	transcriptional regulator; GntR family
NSP-141	SP1451	Cof family protein
NSP-143	SP1466	hemolysin III family pore protein
NSP-145	SP1470	thiamine biosynthesis lipoprotein ApbE; putative
NSP-146	SP1471	oxidoreductase; putative
NSP-147	SP1472	oxidoreductase; putative
NSP-150	SP1491	glycerol uptake facilitator protein, paralog
NSP-151	SP1498	phosphoglucomutase (pgm)
NSP-152	SP1505	membrane protein
NSP-158	SP1567	endoribonuclease L-PSP
NSP-159	SP1586	ATP-dependent RNA helicase; putative
NSP-160	SP1587	oxalate:formate antiporter
NSP-161	SP1591	proline dipeptidase (pepQ)
NSP-163	SP1599	tRNA pseudouridine synthase A (truA)
NSP-164	SP1606	glycosyl transferase; family 2
NSP-165	SP1623	cation-transporting ATPase PacL; putative
NSP-166	SP1633	response regulator
NSP-168	SP1646	metallo-beta-lactamase superfamily protein
NSP-169	SP1651	thiol peroxidase (psaD)
NSP-170	SP1674	phosphosugar-binding transcriptional regulator; putative
NSP-171	SP1682	sugar ABC transporter; permease protein
NSP-173	SP1686	oxidoreductase; Gfo/Idh/MocA family
NSP-174	SP1704	ABC transporter; ATP-binding protein; putative
NSP-175	SP1710	nitroreductase family protein
NSP-176	SP1715	putative ABC transporter; ATP-binding protein
NSP-178	SP1739	KH domain protein
NSP-181	SP1744	iojap-related protein
NSP-182	SP1745	isochorismatase family protein
NSP-183	SP1753	sodium/dicarboxylate symporter family protein
NSP-184	SP1778	water channel protein
NSP-185	SP1780	oligoendopeptidase F; putative
NSP-187	SP1782	ribosomal protein L11 methyltransferase (prmA)
NSP-188	SP1790	ATPase; AAA family
NSP-190	SP1854	galactose operon repressor (galR)
NSP-191	SP1860	choline transporter (proWX)
NSP-192	SP1874	ribosomal large subunit pseudouridine synthase B (rluB)

Table 3. *Continued*

NSP-194	SP1901	RNA methyltransferase; TrmA family
NSP-195	SP1909	oxidoreductase; short chain dehydrogenase/reductase family
NSP-196	SP1918	ABC transporter; ATP-binding protein
NSP-197	SP1943	acetyltransferase; GNAT family
NSP-199	SP1963	putative hemolysin
NSP-200	SP1966	UDP-N-acetylglucosamine 1-carboxyvinyltransferase (murA)
NSP-201	SP1975	stage III sporulation protein J (spoIIIJ)
NSP-203	SP1991	deoxyribonuclease; TatD family
NSP-204	SP1996	universal stress protein family
NSP-205	SP2011	ribosomal large subunit pseudouridine synthase; RluD subfamily
NSP-206	SP2024	pts system; cellobiose-specific iia component
NSP-207	SP2034	hexulose-6-phosphate isomerase; putative
NSP-208	SP2035	hexulose-6-phosphate synthase; putative
NSP-209	SP2038	PTS system; membrane component; putative
NSP-210	SP2039	sakacin A production response regulator
NSP-211	SP2073	ABC transporter
NSP-212	SP2075	ABC transporter; ATP-binding protein
NSP-213	SP2075	ABC transporter, ATP-binding/permease protein
NSP-214	SP2092	UTP-glucose-1-phosphate uridylyltransferase (galU)
NSP-215	SP2095	5-formyltetrahydrofolate cyclo-ligase family protein
NSP-216	SP2096	peptidase; M20/M25/M40 family
NSP-217	SP2112	repressor protein
NSP-219	SP2129	transport protein SgaT; putative (sgaT)
NSP-222	SP2153	peptidase; M20/M25/M40 family
NSP-223	SP2170	adcB protein (adcB)
NSP-224	SP2176	D-alanine-activating enzyme (dltA)
NSP-225	SP2188	chaperonin; 33 kDa (hslO)
NSP-226	SP2189	TIM-barrel protein; putative; NifR3 family
NSP-227	SP2192	histidine kinase
NSP-228	SP2194	ATP-dependent Clp protease; ATP-binding subunit
NSP-229	SP2196	probable sulfate transport atp-binding protein cysa
NSP-230	SP2198	ABC transporter; permease protein
NSP-231	SP2206	ribosomal subunit interface protein (yfiA)

[a]Not listed are 60 hypothetical or conserved hypothetical proteins.

complete one cycle, i.e. from genomic PCR to pneumococcal bacterial transformation result. The yield of pEVP3 clones from any given plate on first run was 60–80%. Some genes required more time than others, adding to the complexity of quantitation of the entire effort. Therefore, to do all 347 knockouts again would require six to seven cycles, or 36–42 person-days. We feel that this is an improvement in time and efficiency over other similar target identification and validation attempts.

Using the pneumococcus, both conserved genes of known function as well as conserved genes of unknown function were disrupted in a systematic fashion to establish *in vitro* essentiality of these individual genes in this organism. In the case of disruptions where the bacteria survived and formed colonies (i.e. non-essential function on an agar plate), the cellular and colony morphologies were examined visually for subtle alterations. Disruption survivors were stocked at –80°C, for potential further examination of the gene disruption by *in vivo* cell culture or animal model infections. In cases where a disruption in the pneumococci resulted in lethality, a secondary construct using a full-length gene was used in the pneumococcal pEVP3 system to test for possible polar effects caused by plasmid integration. This resulted in a gene duplication of the essential gene versus disruption. If lethality was again the result, the gene was considered to be in an essential operon and required additional experimental analysis.

There are examples where a lethal insertion was ascribed to polarity and the downstream target is known to be essential from our own work. One is a cluster of five genes on the *S.pneumoniae* genome (SP1588–SP1592), for which we obtained three disruptions (in all genes except 1588 and 1592). An essential disruption within coding region SP1589, a Mur ligase family protein, was an essential event with a polarity effect (+ polarity). An essential disruption occurred in the gene immediately downstream, SP1590, a conserved hypothetical protein which is essential, but with no polarity issues (– polarity), as a disruption of the next gene, SP1591, was determined to be non-essential. From these data, one can infer that the non-polar status of a gene disruption can be confirmed when the next gene (SP1591) is found to be non-essential. However, the final gene going in the same direction in this cluster, SP1592, may still be non-essential too, since it is possible that SP1592 is essential but has an independent promoter driving it that makes it immune to the polarity of upstream insertions. To be conclusive, it is necessary to further examine the polarity issue by experimentation beyond

Table 4. Functional role of the 113 essential gene disruptions[a]

Cellular role	Subtotal	Total
Amino acid biosynthesis		1
Aspartate family	1	
Biosynthesis of cofactors, prosthetic groups and carriers		4
Thiamine	1	
Pantothenate	3	
Cell envelope		14
Biosynthesis of surface poly/liposaccharides	4	
Biosynthesis of murein sacculus and peptidoglycan	10	
Cellular processes		7
Chaperones	1	
Cell division	2	
Protein and peptide secretion	4	
DNA metabolism		11
DNA replication, recombination and repair	9	
Chromosome-associated proteins	2	
Energy metabolism		2
Pentose phosphate pathway	1	
Glycolysis/gluconeogenesis	1	
Fatty acid, lipid and sterol metabolism		10
Biosynthesis	10	
Protein synthesis		2
tRNA aminoacylation	2	
Purines, pyrimidines, nucleosides and nucleotides		4
Purine ribonucleotide biosynthesis	1	
Sugar-nucleotide biosynthesis and conversions	3	
Regulatory functions		6
General	4	
Protein interactions	2	
Transcription		2
Transcription factors	2	
Translation		9
Translation factors	5	
Degradation of proteins, peptides and glycopeptides	1	
Other	3	
Transport and binding proteins		4
Amino acids, peptides and amines	4	
Unknown		37
Unknown	37	
Total		113

[a]Categories are based on those proposed by Riley (32).

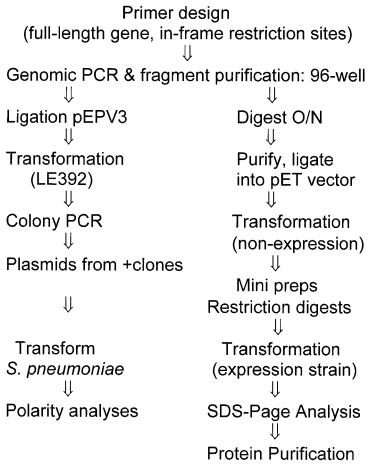


Figure 3. Scheme for overexpression of target proteins and polarity analysis. A similar high-throughput 96-well microtiter plate approach was used. The regulatable pET vector system was used for protein overexpression.

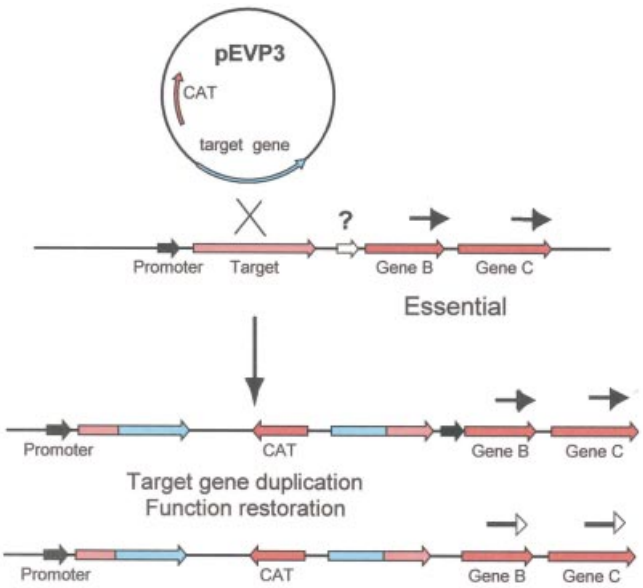


Figure 4. Use of plasmid pEVP3 for polarity analysis of pneumococcal genes. The full-length target was cloned into this vector, which is incapable of replication in pneumococci. Selection for the insertion–duplication event was on medium containing chloramphenicol. Arrows indicate direction of transcription. The question mark indicates the possible position of an additional promoter.

our polarity check method. Such polarity analyses could result in the identification of multiple essential genes within the operon, thus adding genes to the list. Of the 113 genes listed where gene disruptions could not be obtained, 57 were determined to have no issues of polarity (Table 2). These genes are either not in operons, are the last gene in an operon or are in an operon with no downstream essential genes. For these 57 genes lacking polarity effects, as well as the 113 genes found to be non-viable upon disruption, the majority could be assigned a clear or putative role, but roughly one-third were of unknown function (Table 4). Where known, the functions of the 113 conserved essential gene products encompassed a broad variety of cellular processes, including cell wall biosynthesis, DNA replication, cell division and fatty acid biosynthesis, among others (Table 4). Most of these targets have yet to be exploited for drugs in human clinical use.

With the development of this high-throughput gene disruption approach, we have rapidly and systematically assessed the essentiality of the computer-generated list of conserved genes of both known and unknown function. An added value of the gene disruption system described here is that a similar high-throughput approach was successfully used to evaluate expression of gene products and to assess polarity issues. Genes of known function that can be assayed and are proved essential have become immediate candidates for further development as high-throughput screens using their protein products. Unknown function genes provide a greater challenge and require multiple strategies aimed at deducing function and generating a screening system. The expectation is that

genomic approaches will identify a significant number of new drug targets (31), which will lead to the discovery of several novel agents for the treatment of bacterial infections.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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