Functional Selection of Vaccine Candidate Peptides from *Staphylococcus aureus* Whole-Genome Expression Libraries In Vitro

Thomas Weichhart,[1] Markus Horky,[1] Johannes Söllner,[1] Susanne Gangl,[1] Tamàs Henics,[1] Eszter Nagy,[1] Andreas Meinke,[1] Alexander von Gabain,[1] Claire M. Fraser,[2] Steve R. Gill,[2] Martin Hafner,[1] and Uwe von Ahsen[1]*

Intercell AG, 1030 Vienna, Austria,[1] and The Institute for Genomic Research, Rockville, Maryland 20850[2]

An in vitro protein selection method, ribosome display, has been applied to comprehensively identify and map the immunologically relevant proteins of the human pathogen *Staphylococcus aureus*. A library built up from genomic fragments of the virulent *S. aureus* COL strain (methicillin-resistant *S. aureus*) allowed us to screen all possible encoded peptides for immunoreactivity. As selective agents, human sera exhibiting a high antibody titer and opsonic activity against *S. aureus* were used, since these antibodies indicate the in vivo expression and immunoreactivity of the corresponding proteins. Identified clones cluster in distinct regions of 75 genes, most of them classifiable as secreted or surface-localized proteins, including previously identified virulence factors. In addition, 14 putative novel short open reading frames were identified and their immunoreactivity and in vivo mRNA expression were confirmed, underscoring the annotation-independent, true genomic nature of our approach. Evidence is provided that a large fraction of the identified peptides cannot be expressed in an in vivo-based surface display system. Thus, in vitro protein selection, not biased by the context of living entities, allows screening of genomic expression libraries with a large number of different ligands simultaneously. It is a powerful approach for fingerprinting the repertoire of immune reactive proteins serving as target candidates for active and passive vaccination against pathogens.

Staphylococcus aureus is a gram-positive bacterial pathogen that causes diseases ranging from minor skin infections to life-threatening deep infections such as pneumonia, endocarditis, meningitis, osteomyelitis, postoperative wound infections, septicemia, and toxic shock syndrome. Hospitalized patients are at particular risk, and the emergence of drug resistance has made many of the available antibiotics ineffective. Approximately 40 to 60% of staphylococcal isolates are resistant to multiple antibiotics, including methicillin and vancomycin. The need for new drugs in order to treat *S. aureus* infections is unmatched with the current situation, as only a limited number of antibiotics have received market approval in the last decades. Passive or active immunization might therefore provide a solution to this problem; however, still no effective vaccine against *S. aureus* is available. Consequently, to produce an effective vaccine it is utterly important to find antigens that raise the production of protective antibodies. Specifically, proteins conserved among different isolates are well-suited for the development of defined vaccines, as nonprotein compounds like polysaccharides often vary among isolates, frequently even specify a serotype, and also fail to induce a long-lasting immune response, except for the more costly conjugate vaccines.

More recently, whole-genome, bioinformatic-based approaches have been reported (21, 27). Proteins from *Neisseria meningitidis* and *Streptococcus pneumoniae* predicted to be cell surface localized or secreted have been expressed as recombinant proteins and empirically tested in animal models. This approach is highly dependent on proper gene identification and annotation and is difficult to apply to the roughly 40% of genes, on average, to which no function can be assigned in a given genome (19). Another intrinsic shortcoming is that it is not known how many of these proteins are actually expressed in vivo during an infection and are able to induce an immune response in humans. Both aspects are requirements for effective vaccines. A functional selection to identify proteins with these characteristics would therefore be advantageous.

The human humoral immune system is able to raise a specific antibody profile upon encountering a given pathogenic microorganism. These antibodies can be seen as an inverse, immunological blueprint suitable to identify the corresponding proteins out of proteomic samples or expression libraries. A number of these identified proteins from *S. aureus* were shown to induce antibodies that possess antistaphylococcal activity (2–4, 25). Thus, the comprehensive identification of the antibody repertoire against a given pathogen is a prerequisite for an effective vaccine and, moreover, could give more insight into the pathogenesis of a particular infection.

In recent years, several in vitro protein selection technologies have emerged (8, 9, 17, 22) that, by circumventing the need to introduce the encoding DNA of interest into a cellular host, provide two very distinct main properties. First, they allow access to library complexities that are basically limited only by the amount of DNA that is added to cell-free protein synthesis systems, thereby exceeding in vivo display and selec-

* Corresponding author. Mailing address: Intercell AG, Campus Vienna Biocenter 6, 1030 Vienna, Austria. Phone: 43 1 20620-220. Fax: 43 1 20620-800. E-mail: uahsen@intercell.com.

tion systems by several orders of magnitude. Second, virtually any peptide or protein that can be produced by the ribosomal machinery can be displayed and selected for, because the in vitro environment excludes any interference with a host's physiology (e.g., toxic proteins and membrane penetration).

Here we present the application of functional in vitro protein selection technology for identification of immunoreactive peptides on a genomic scale for the first time. Using an *S. aureus*-derived genomic library, in vitro selection was successfully performed with high-titer sera against *S. aureus*, thereby expanding in vitro protein selection also for the simultaneous use of a multiple ligand set. Emphasis was set to characterize the selection dynamics and to confirm the immunogenicity of the identified antigens by peptide enzyme-linked immunosorbent assay (ELISA). Finally, an expression comparison with an in vivo based surface display system was performed.

MATERIALS AND METHODS

Template design. The following oligodeoxyribonucleotides were used for PCR to generate DNA templates for in vitro selection: ICC277 (5′-CGAATAATAC GACTCACTATAGGGAGACCACAACGGTTTCCCACTAGTAATAATTT TGTTTAACTTTAAGAAGGAGATATATCCATGCAGACCTTGGCCGGC CTCCC-3′), which introduces T7 RNA polymerase promoter, 5′ stem loop, and a ribosome binding site, and ICC202 (5′-GGCCCACCCGTGAAGGTGAGCC GGCGTAAGATGCTTTTCTGTGAC-3′), which introduces a 3′ stem loop structure. These primers amplify *myc9* or *S. aureus* genomic fragments ligated into vector pMAL4.1 (4) with three glycines as linker and the first 89 amino acids from β-lactamase (*bla*) as tether. A 390-bp DNA sequence encoding nine copies of the myc epitope was ligated into the *Sma*I restriction site of pMAL4 (pMAL4 contains an additional guanine between the *Sma*I and *Not*I site compared to pMAL4.1, thereby restoring the reading frame of *bla*). From this vector myc9-bla was constructed by PCR amplification. The construction of the genomic *S. aureus* library was described previously (4). Plasmid DNA from pMAL4.1 containing frame-selected genomic regions was used as the template for PCR. The Expand High-Fidelity PCR system kit (Roche) was used for all PCRs.

In vitro selection. One selection cycle was carried out with the following modifications to the described procedure (7): in vitro transcription was performed with RIBOMAX T7 large-scale RNA production systems (Promega) as suggested by the manufacturer, and RNA was purified with YM-100 Microcon columns (Amicon). A 22-µl in vitro translation reaction contained 2 µg of RNA, 4.4 µl of Premix Z, 200 mM potassium glutamate, 13.8 mM magnesium acetate, 8 µl of *Escherichia coli* S-30 extract (2.6 µg of protein/µl), and 1.9 mM methionine and was carried out for 9 min at 37°C. Anti-*ssrA* oligonucleotide (8) was omitted, as it did not show any obvious effect in our selection of genomic DNA fragments (data not shown). Immunoprecipitation of the ribosomal complexes was accomplished with MAGmol Protein G Microbeads (Miltenyi Biotec). The translation reaction mixture was transferred to a prechilled tube containing 35.2 µl of 10% milk–WBT buffer [WBT buffer is 50 mM Tris(Ac)$_2$ (pH 7.5), 150 mM NaCl, 50 mM Mg(Ac)$_2$, 0.1% Tween 20] mixed with 52.8 µl of WBTH buffer (WBT buffer and heparin [2.5 mg/ml]), 1 µg of 9E10 myc antibody, or 10 µg of *E. coli* cell, and extract-preadsorbed high-titer immunoglobulins G (IgGs) were used as indicated. Serum selection and treatment was performed as described previously (4). After incubation on ice for 90 min, 50 µl of protein G Microbeads were added, and incubation was prolonged for another 90 min. The immunoprecipitate was applied to a precooled microcolumn (Miltenyi Biotec), washed five times with ice-cold WBT buffer, and incubated with 20 µl of EB20 elution buffer [50 mM Tris(Ac)$_2$ (pH 7.5), 150 mM NaCl, 20 mM EDTA, *Saccharomyces cerevisiae* RNA (50 µg/ml; Sigma)] for 5 min at 4°C. Elution was completed by eluting with 100 µl of EB20. Reverse transcription (RT)-PCR was performed with Superscript II (Roche Diagnostics). After four rounds of selection the library fragments were cloned into the transcriptionally silenced vector pMAL4.34 (Hanner et al., unpublished data) with *Fse*I and *Not*I, selected with ampicillin and kanamycin, and sequenced.

Peptide ELISA and Western blotting. Peptides were synthesized according to their deduced amino acid sequence by 9-fluoroenylmethoxy carbonyl chemistry. ELISA and Western blotting were performed as described previously (4). Values above double the background plus 1 standard deviation (optical density at 450 nm = 0.2) were calculated as positive in ELISA. Numbers in Table 3 were calculated from ELISA values as follows: <0.2 = −; <0.5 = 1; <0.8 = 2; >0.8 = 3. FhuA expression analysis was performed as described previously (5).

Semiquantitative PCR. Equal amounts of cDNA template from the individual rounds and an appropriate amount of the input library were used in a PCR with primers ICC277 and ICC202. After analysis by gel electrophoresis and photometry, the PCR products were used as templates for semiquantitative PCR with 30 cycles. ICC509 (5′-CACCCAACTGATCTTCAGC-3′) hybridizing 40 bp downstream of the *Sma*I site in the *bla* gene was used as the antisense primer in all reactions. The following sense primers were utilized to amplify specific regions of the open reading frames (ORFs): 5′-TATGTTTATAACGATGGGA-3′ for SA0021, 5′-GTTGGTCGTAAATACGGGC-3′ for SA0195, 5′-CGTGCAATTAT AGCTATTG-3′ for SA0200, 5′-TTCACAGTCTTAGCAATTG-3′ for SA0334, 5′-GTAGTTAGCATTGCGACGG-3′ for SA0835, 5′-ACAGAAGACCTAAATTTC C-3′ for SA1777, 5′-TTGATGGCGTTCATGCACC-3′ for SA2659, and 5′-AATG GGTTATCGGTCAATG-3′ for SA2702.

RT-PCR. The following primers were used for RT-PCR: IsaA sense, 5′-GCG CTGAAGTAAACGTTGATCAAGCA-3′; IsaA antisense, 5′-TAGAATCCCC AAGCACCTAAACCTTG-3′; PSAN1 sense, 5′-TGTGCTATTATTCCCCCTA TTG-3′; PSAN1 antisense, 5′-TGGTAGGTTAGTACAAGGAG-3′; SAN1 sense, 5′-GTCCATGGAAGTTACCACC-3′; SAN1 antisense, 5′-GTGGCAGC GCGTATGC-3′; SAN2 sense, 5′-CAGTGTTTCAATAATCAGCCAAC-3′; SAN2 antisense, 5′-AGCTGATGCATTTTGCTTTACC-3′; SAN3 sense, 5′-AT CAGGTGTGTGATGATAATCC-3′; SAN3 antisense, 5′-GAAAAAATTAAC AGCAGCAGCG-3′; SAN4 sense, 5′-GCTTTTGCGACATTAGCGC-3′; SAN4 antisense, 5′-AAAACTTAAACAGTTGAGAGATGC-3′; SAN5 sense, 5′-AAT CTTTTGATGTTTCATTTTCAACTG-3′; SAN5 antisense, 5′-GCCTAAATTA GTTATTCTAATGGG-3′; SAN6 sense, 5′-GCCAGTCACTTCTCGTTC-3′; SAN6 antisense, 5′-CGGTTGAATGCCATGTAAAAGC-3′; SAN7 sense, 5′-T ATAGCATTTACCGCTTGATTTG-3′; SAN7 antisense, 5′-CAGACAACAAG CTATTGAAAG-3′; SAN8 sense, 5′-TAATCAAAACTGTCACCTTTATCAG-3′; SAN8 antisense, 5′-CGGAGGTCGAATAACATGC-3′; SAN9 sense, 5′-TT GAGTTTGTGCGTTTGCTG-3′; SAN9 antisense, 5′-ATGCCAATACACAGC CTAAC-3′; SAN10 sense, 5′-ATGCTTTTGAGTTACTTCATAATAC-3′; SAN10 antisense, 5′-AGAACATCGGTTATCATTTTTTAAAC-3′; SAN11 sense, 5′-TGT GTACCTATGAATTGTTGCG-3′; SAN11 antisense, 5′-CCTGATGATATTTAT GTCAACAATG-3′; SAN12 sense, 5′-GATTGACTGCCACCACCG-3′; SAN12 antisense, 5′-CATTCTACATCATCAAGTAATAACC-3′; SAN13 sense, 5′-CATC TGGGTGAACTTGAACAG-3′; and SAN13 antisense, 5′-GATATCCCTAATGA AACAACTGG-3′. RNA was extracted from an *S. aureus* COL strain (optical density at 600 nm = 1.7 [BHI medium]) as described previously (13) and used for RT (Superscript II; Roche Diagnostics). Two microliters of cDNA was used for PCR with 25 to 30 cycles (94°C for 30 s, 42°C for 30 s, 72°C for 2 min). As a negative control, an appropriate amount of RNA was directly used for PCR. The amplified products were cloned and confirmed by sequencing.

Bioinformatic analysis. The localization of the selected clones was determined by BLAST analysis against the *S. aureus* COL genome (http://www.tigr.org/tigr -scripts/CMR2/CMRHomePage.spl) (20) as well as ORF set on a local BLAST server and the localization data stored in a Sybase relational database. TM/S prediction of transmembrane domains or signal peptides was done with the following programs: TMpred (http://www.ch.embnet.org/software/TMPRED-_form.html) (10) and SignalP V1.1 (http://www.cbs.dtu.dk/services/SignalP/index.html) (18).

RESULTS

In vitro selection of antigens by ribosome display. In order to adapt the ribosome display method (8) for selection of immunoreactive peptides, we first employed a well-characterized antibody-epitope system. Nine consecutive repeats of the myc epitope were cloned in frame with a fragment of the β-lactamase gene serving as a tether, interspersed by a flexible glycine linker to facilitate independent folding of the polypeptide of interest. After a number of modifications and optimization steps (see Materials and Methods), we were able to specifically select and enrich the myc-containing sequence very efficiently and dependent on myc antibody presence. A sample containing a 10^6-fold molar excess of an unrelated RNA over the myc-containing RNA was subjected to three rounds of selection and amplification in the presence of myc antibody.
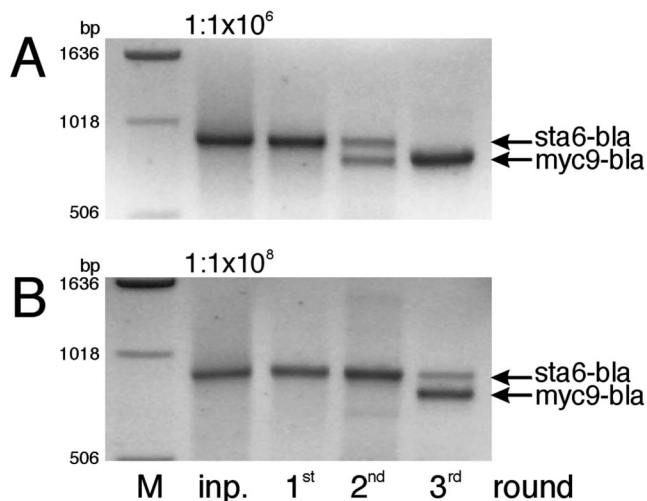
FIG. 1. Ribosome display with antibodies as selective agents. RNA encoding the *myc* epitope sequence was mixed with an unrelated fragment (*sta6-bla*) at a dilution of 1:10[6] (A) or 1:10[8] (B). Three selection cycles were performed with antibodies against the *myc* epitope. Shown are PCR products of the input mix (inp.) and the cDNA of the three selection cycles as indicated. On the left the length of the DNA marker (lane M) is depicted.

Figure 1A shows that only the myc-containing DNA fragment was present after the third round of selection. At a dilution of 1 to 1×10^8, the myc DNA fragment is dominant over the unrelated one after only three rounds of selection (Fig. 1B).

Note that this in vitro expression and selection system has the intrinsic capability to screen libraries with complexities exceeding 10^{12} members; here it was sufficient to demonstrate the sensitivity of our system to screen rather low complexity libraries required for covering microbial genomes.

For the actual selection of antigenic regions from *S. aureus* we transferred a previously described genomic library derived from the methicillin-resistant *S. aureus* strain COL (4) into the ribosome display context by PCR. The IgG fraction from pre-characterized sera from healthy individuals exhibiting a high titer and opsonizing activity against *S. aureus* as previously described was used for selection (4). After four rounds of selection, the resulting pool was cloned into a transcriptionally silenced vector and sequenced. As a first confirmation on the immunoreactivity of the selected pool, immune precipitation experiments with six different clones that were frequently found revealed that they all precipitated with sera used for selection but not with infant serum as a negative control (data not shown).

Bioinformatic analysis. Based on sequence determination from 1,576 selected clones, bioinformatic analysis revealed the following picture.

(i) Selected clones cluster in distinct regions. Selected clones cluster in distinct regions of 75 different ORFs in an overlapping fashion (Fig. 2A). A number of control PCRs with primers matching ORF regions other than the identified ones revealed that these regions were indeed present in the input library but evidently were not selected and enriched (Fig. 2A
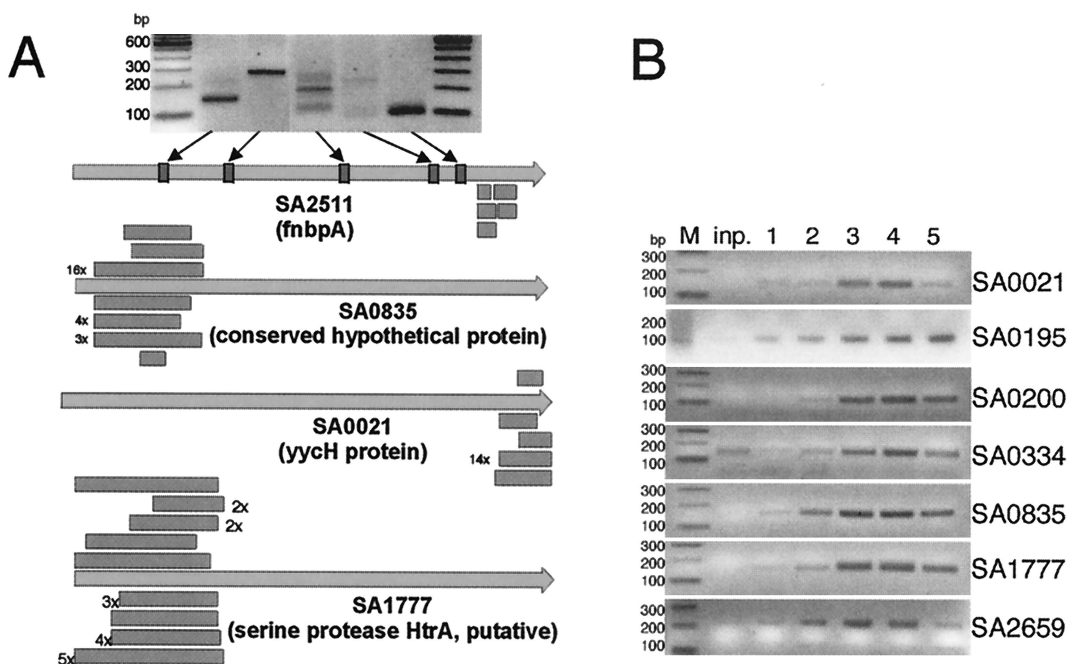
FIG. 2. Evaluation of antigenic epitopes. (A) Alignment of recovered epitopes to individual ORFs from *S. aureus*. SA2511 (coding for fibronectin-binding protein, FnbpA), SA0835, SA0021, and SA1777 are shown as examples. For *fnbpA*, PCR products amplified from the input library are shown. The sense primer binds to the indicated regions of *fnbpA*, whereas the antisense oligonucleotide hybridizes to the constant region of the β-lactamase spacer. (B) Enrichment of frequently found *S. aureus* library fragments during the selection process. Enrichment was determined by semiquantitative PCR with clone-specific primers. The sense primer hybridizes to the indicated *S. aureus* library fragment (compare Table 1). The antisense primer binds to the constant β-lactamase spacer. Each lane shows the PCR product of a different template: inp., DNA of input library; 1 to 5, cDNA of the first through fifth round, respectively, of ribosome display screened with normal high titer serum. Marker (M) length is shown on the left.

TABLE 1. Immunoreactive *S. aureus* genes identified in this study[a]

TIGR locus	Gene name or function	No. of clones[b]	Reactivity[c]		IP[d]	% Conservation[e]	TM/S[f]	Region(s)[g] (amino acid)
			P sera	N sera				
SA0006	DNA gyrase, A subunit (*gyrA*)	5	9/14	15/19	ND	81.08	ND	89–130
SA0021	yycH protein (*yycH*)	53			ND	100.00	ND	8–24, 394–494
SA0050	Methicillin-resistant surface protein (*pls*)	6			+	54.05	ND	1191–1255, 1389–1496
SA0103	Drug transporter, putative	8	14/14	18/19	ND	100.00	ND	84–100
SA0195	Maltose ABC transporter, permease protein	100	14/42	4/32	+	100.00	ND	78–129
SA0200	Phosphoglycerate transporter family protein	379	42/42	26/32	+	94.59	ND	323–345
SA0317	Lipase precursor, interruption	33	2/42	10/32	ND	75.00	ND	54–92
SA0334	Conserved hypothetical protein	168			+	100.00	TM/S	1–43
SA0754	Multidrug resistance protein	3		5/5	ND	70.27	ND	155–175
SA0835	Conserved hypothetical protein	65	40/42	30/32	+	100.00	TM/S	6–54
SA0856	Clumping factor A (*clfA*)	6		2/5	ND	21.62	ND	87–98
SA0946	Conserved hypothetical protein	5		2/5	ND	75.68	TM/S	105–122
SA0950	Na+/H+ antiporter, MnhF component (*mnhF*)	66		2/5	ND	66.67	ND	1–21
SA1045	Iron compound ABC transporter	10		5/5	ND	100.00	ND	1–18
SA1062	Bifunctional autolysin (*atl*)	19	8/14	13/18	ND	100.00	ND	64–81
SA1112	Conserved hypothetical protein	349	14/14	18/18	ND	ND	−/−	13–37
SA1138	Cell wall surface anchor family protein	4			ND	ND	ND	53–73
SA1168	Fibrinogen-binding protein (*fib*)	3			ND	100.00	ND	127–141
SA1259	Conserved hypothetical protein	13		2/5	ND	ND	TM/S	196–218
SA1743	Amino acid permease	9	14/14	17/18	ND	94.59	ND	127–152
SA1777	Serine protease HtrA, putative	73	17/42	9/32	ND	100.00	ND	1–30
SA1806	Cell wall surface anchor family protein	9	7/14	16/18	ND	100.00	ND	508–523
SA1919	Transcriptional regulator	3	13/14	18/18	ND	100.00	ND	120–135
SA2125	Peptidase, M20/M25/M40	3	13/14	18/18	ND	97.30	ND	293–312
SA2347	Drug resistance transporter, EmrB/QacA subfarr	5	14/14	18/18	ND	100.00	ND	205–224
SA2437	Bicyclomycin resistance protein (*bcr*)	8	13/14	18/18	ND	100.00	ND	54–104
SA2505	Cell wall surface anchor family protein	5	19/42	7/32	+	ND	ND	829–849
SA2511	Fibronectin-binding protein A (*fnbA*)	8	13/14	12/18	ND	100.00	ND	129–196, 876–913
SA2652	Clumping factor B (*clfB*)	17	41/42	32/32	ND	100.00	ND	711–751
SA2659	Zinc metalloproteinase aureolysin	12	4/42	1/32	ND	97.30	ND	90–107

[a] Listed is a representative subset of 75 genes for which three or more independent clones were identified as mapping to the same region. The Institute for Genomic Research (TIGR) locus and gene name are according to the CMR database (20).

[b] Number of clones located in the respective gene.

[c] Reactivity in peptide ELISA with patient (P) sera or normal-subject high-titer (N) sera (as described in reference 4). Data are presented as number of serum samples positive for reactivity/total number of serum samples tested.

[d] Immunoprecipitation reaction: +, positive; ND, not determined.

[e] Conservation of respective genes in 36 *S. aureus* isolates as determined by DNA chip hybridization (6). ND, not determined.

[f] Prediction of transmembrane domain or signal peptide (TM/S) (ND, not determined).

[g] Selected region(s) in relative amino acid position.

and data not shown). A representative subset of identified genes can be seen in Table 1.

(ii) Specific enrichment of selected genes. Shown in Table 2 is the specific enrichment of selected genes allocated to functional classification (TIGRFAM) (20) in comparison to its percentage present in the unselected whole genome. The majority of the identified ORFs can be assigned to cell envelope function (25%), transporter proteins (24%), or pathogenesis- or toxin-involved functions (9%), among them those described to provide protective activity in challenge models (coding for clumping factor A [*clfA*], fibronectin-binding protein A [*fnbpA*] and fibrinogen-binding protein [*fib*]) (12, 14, 23) (Table 1). This result underscores the above-mentioned notion that a functional screen using human sera as selective agent should identify proteins accessible to the immune system and expressed during an infection in vivo. In addition, most selected genes with no assigned function (encoding hypothetical proteins) were found to contain a predicted signal sequence required for export and/or a *trans*-membrane domain typical for membrane associated proteins, thereby extending the above notion also into this group (Table 1).

(iii) Variation in number of clones matching a particular ORF. The number of clones matching a particular ORF varies considerably, most probably reflecting corresponding antibody abundance and/or affinity (4). The relative identification frequency of the antigens was dependent on the selection round (see below).

(iv) Orientation of clone clusters. Several clone clusters fall into the orientation opposite that of annotated genes. The transcriptional activity from these clusters and a brief characterization are described below and in Table 4. However, it emphasizes the annotation-independent true genomic nature of our approach and reveals completely new putative targets for vaccine development.

(v) Gene conservation among 36 *S. aureus* isolates. A comprehensive study on gene conservation among 36 *S. aureus* isolates was recently performed by DNA chip hybridization technology (6). Of the immunodominant ORFs identified in our study, 66% were present in all of the 36 different strains tested and 82% of the ORFs were conserved in more than 90% of the strains (Table 1).

Repetition of the selection with a similar serum set revealed

TABLE 2. Grouping of genes identified in this study into functional categories according to the CMR database[a]

Main cellular role	Cellular subrole	In vitro selection		Whole genome	
		No.	%	No.	%
Cell envelope	Biosynthesis and degradation of surface polysaccharides and lipopolysaccharides	3	4.00	43	1.44
	Other	16	21.33	74	2.47
Total		19	25.33	170	5.69
Cellular processes	Cell division	1	1.33	18	0.60
	Pathogenesis	4	5.33	58	1.94
	Toxin production and resistance	3	4.00	87	2.91
Total		8	10.67	*206*	6.89
Hypothetical proteins	Conserved	10	13.33	594	19.87
	Other	3	4.00	403	13.48
Total		13	17.33	997	33.34
Protein fate	Degradation of proteins, peptides, and glycopeptides	3	4.00	46	1.54
	Protein and peptide secretion and trafficking	1	1.33	18	0.60
Total		4	5.33	88	2.94
Transport and binding proteins	Amino acids, peptides, and amines	4	5.33	61	2.04
	Anions and cations	4	5.33	86	2.88
	Carbohydrates, organic alcohols, and acids	3	4.00	38	1.27
	Other	7	9.33	36	1.20
Total		18	24.00	290	9.70
Other Total		13	17.33	1,239	41.44
Grand Total		75	100.00	2,990	100.00

[a] Genes were grouped according to the CMR database (TIGRFAM) (20) in comparison to the number of genes and respective percentages in the genome. A small number of genes fit into more than one classification group (compare CMR [http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl]).

a largely overlapping set of ORFs, indicating the reproducibility of the screen (data not shown).

Selection dynamics of the identified clones. In order to visualize the enrichment of frequently identified clones during the selection cycles, semiquantitative PCRs using DNA fragment-specific primers were performed. Input DNA library as well as DNA pools from the consecutive rounds of selection was used as a template. Examples of the gradual enrichment of individual fragments are shown in Fig. 2B. Interestingly, the dynamics and competitive nature of selection for individual fragments is visible. The peak amount of DNA from a number of clones can be seen in rounds three and four, leveling off in the fifth round (ORFs SA0021 and SA2659). Sequencing a number of clones from the third round of selection revealed a wider variety of different clones with low frequency. In the fifth round, however, the pool is dominated by very few clones derived from ORF SA0195 and SA0200 (data not shown). This indicates that at levels where the selection has advanced to the stage that every synthesized peptide—at limiting ribosomes—is able to interact with its cognate antibody, these peptides outcompete others dependent on antibody abun-

dance and/or affinity. This selection dynamic is also observed in in vitro selection of nucleic acids (26).

Serological confirmation of selected peptides. Next, peptides corresponding to the identified regions of 25 more or less frequently selected clones were synthesized and tested by peptide ELISA. A large fraction of sera from healthy individuals exhibiting a high titer against *S. aureus* as well as from patients suffering from an *S. aureus* infection showed reactivity towards the selected peptides (Tables 1 and 3). This demonstrates that the identified peptides interact with the selective agent independently of the ribosome display context. However, the identification of immunodominant peptides is highly dependent on the choice of serum used. This is best exemplified with the aureolysin-derived peptide (Table 3 [SA2659]). It reacts strongly only with one of the sera used in the serum pool for selection but not with any of the other normal high-titer sera tested.

The presence of specific antibodies is molecular proof for the expression of the corresponding antigens in vivo during exposure, colonization, or disease. Many of the epitopes identified have corresponding antibodies in both healthy-subject

TABLE 3. Results of ELISA with synthesized peptides[a]

ORF name	Position (amino acids)	Healthy-subject high-titer serum																															
		3*	4*	9*	15*	35*	2	5	6	7	8	10	11	14	16	17	18	19	20	21	24	26	28	29	31	32	34	36	37	38	39	40	41
SA0006	114–130	2	2	2	—	ND	1	—	1	1	1	1	1	—	3	1	2	1	1	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA0103	84–100	3	2	2	2	ND	2	1	2	2	3	3	2	1	2	2	2	2	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA0195	78–128	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1
SA0200	323–345	1	—	2	1	2	2	1	1	1	2	1	1	1	1	1	1	1	—	—	1	1	—	1	1	1	—	1	1	1	—	1	1
SA0317	147–162	—	1	1	—	—	1	1	—	1	1	—	1	—	—	—	—	—	—	—	1	—	—	—	1	—	1	—	1	—	—	—	—
SA0835	6–54	3	1	2	1	1	3	1	2	2	2	2	1	1	3	2	2	1	1	1	2	1	1	1	1	—	2	1	1	1	—	2	2
SA1062	63–81	2	—	2	2	ND	1	—	2	1	2	2	2	1	2	2	—	2	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA1112	13–37	2	3	2	2	ND	2	1	2	2	2	2	2	3	2	2	2	2	2	1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA1743	127–152	2	1	2	2	ND	1	1	2	2	2	2	2	1	2	2	—	2	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA1777	13–29	—	—	1	—	—	1	—	—	1	1	—	1	—	—	—	—	—	—	—	1	—	1	—	1	—	1	—	1	—	—	—	—
SA1919	120–135	2	3	2	2	ND	2	1	3	2	2	3	2	3	2	3	2	3	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA2125	293–312	3	3	3	2	ND	2	2	3	2	2	2	3	2	3	2	2	2	2	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA2347	205–224	3	2	2	2	ND	2	1	2	2	2	3	2	1	2	2	2	2	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA2437	83–102	3	3	3	2	ND	2	1	3	2	3	2	3	2	3	2	3	3	3	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA2505	824–832	2	—	—	—	—	—	—	—	—	—	1	—	—	—	1	1	—	—	—	—	—	—	—	—	—	—	—	—	—	1	1	2
SA2511	131–148	2	—	1	1	ND	—	—	1	—	2	2	1	—	1	1	1	1	1	—	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
SA2652	601–617	1	1	2	2	3	2	1	1	1	1	1	1	1	2	2	2	2	2	1	2	2	2	1	2	2	2	2	2	2	2	2	3
SA2659	90–107	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
SAN7	1–18	1	—	2	1	2	2	1	1	1	1	1	1	1	1	1	1	2	—	1	1	2	1	1	1	1	1	1	2	2	1	—	2
SAN10	27–35	2	2	1	1	2	1	—	1	2	—	1	1	1	—	1	1	1	1	1	—	1	1	1	1	1	1	1	1	—	1	—	1

[a] Peptides synthesized from the deduced amino acid sequence of the selected epitopes were tested in ELISA with healthy-subject high-titer and patient sera. On the left the ORF name and the amino acid region tested are shown. The sera indicated with an asterisk were used in the selection procedure. Increasing reactivity (indicated as 1, 2, or 3) with the serum was tested as described in Materials and Methods. —, no reactivity; ND, reactivity not determined.

and patient sera. However, since these different encounters with the human host require different virulence factors (adhesins and toxins) and other bacterial components, it is not surprising that the antigen pattern revealed by sera from healthy individuals with high titers of antibodies (nasally colonized and noncolonized) and from patients with different *S. aureus* infections varies. Indeed, we have identified epitope-specific antibodies, which seemed to be more prevalent—meaning higher antibody levels and/or more-reactive sera were observed—in one of the two donor populations. For example, SA0317 mainly reacted with the sera with high titers of antibody, whereas SA0195 and SA2505 reacted more frequently with individual patient sera.

Characterization of newly identified putative short ORFs. Fourteen clone groups are located in the opposite orientation to annotated genes, indicating the presence of putative antisense transcripts, which could encode immunodominant peptides. Analysis of the surrounding areas revealed possible ORFs with sizes varying between 21 and 167 amino acids in length taking into account the closest start (including TTG codons) and stop codon in frame with the selected peptide (Table 4). BLAST analysis of these putative ORFs against the nonredundant database did not reveal any considerable similarity to any annotated sequence (data not shown).

All peptides derived from these clones that were tested in peptide ELISA reacted strongly with a variety of sera (Tables 3 and 4), to an extent similar to that observed with peptides derived from annotated genes. However, we cannot exclude the possibility of cross-reactive antibodies in the sera. To confirm the actual in vivo transcription of these putative short ORFs, total mRNA samples from *S. aureus* grown in BHI medium were used for mRNA expression analysis. RT-PCR analyses with primers specific for corresponding short ORFs were performed and confirmed the mRNA presence for most

putative ORFs that were tested (Fig. 3 and Table 4). Northern analysis with five strand-specific single-stranded probes revealed the existence of mRNA transcripts (Table 4 and data not shown).

In vivo expression analysis of in vitro-selected peptides. Finally, we wanted to experimentally verify the theoretical advantage of this in vitro expression and selection system (ribosome display) for displaying a variety of genomically encoded peptides. Certain peptides might interfere with the physiology in general or cannot be presented in an in vivo-based display system. Clones corresponding to 19 different ORFs that were identified in the in vitro selection were transferred to an FhuA-based surface display system in *E. coli* (5). The resulting clones were grown and protein extracts were taken. The expression and stability of the FhuA fusion proteins were tested with an FhuA-specific antibody and Western analysis. As can be seen in Fig. 4, only 6 out of 19 clones tested show full-length FhuA fusion protein, probably indicating that the majority of fusion proteins cannot be displayed on the surface of *E. coli* and may therefore be rapidly degraded in the cytoplasm or periplasm. In additional experiments, more clones were tested, and approximately 55% of clones that were identified by ribosome display did not show reactivity in Western blots when expressed in the FhuA platform. A similar percentage of reactivity was observed upon testing genomically encoded peptides from *Streptococcus pyogenes* (data not shown), indicating the general nature of this phenomenon.

DISCUSSION

In this study we describe the identification of novel antigens from the pathogen *S. aureus* by in vitro protein selection. For the first time we show that ribosome display allows the simultaneous selection for a set of interacting proteins from

TABLE 3—*Continued*

	Patient serum																																									
1	2	3	4	5	6	7	8	9	15	17	18	19	20	21	22	23	24	25	26	27	30	31	32	33	34	35	36	37	38	39	42	44	45	47	48	49	50	51	52	53	54
—	2	1	1	—	1	1	1	—	—	2	1	—	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1	2	1	2	1	2	2	2	2	1	2	3	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
—	—	—	1	2	—	—	—	—	—	1	2	2	1	—	1	—	—	2	1	—	—	—	2	—	—	—	2	—	—	—	—	1	—	—	—	1	—	—	1	—	
1	3	1	3	3	1	1	2	2	1	2	2	3	2	1	2	1	2	3	2	1	1	1	3	1	3	1	2	1	1	1	1	2	2	1	2	1	3	1	2	1	2
—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	3	1	2	1	1	1	2	1	1	—	1	1	3	2	1	1	1	2	2	2	1	—	1	1	2	1	1	1	1	2	3	2	2	1	2	2	3	3	1	1	1
1	2	—	2	—	2	1	2	1	—	1	2	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	3	1	3	2	3	2	3	2	2	2	3	3	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	3	1	3	2	2	2	3	2	2	2	3	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
—	—	—	1	1	1	—	1	—	—	3	1	2	—	1	—	—	1	3	—	—	—	1	—	3	—	—	—	—	—	1	1	—	—	—	—	3	1	1	—	—	
2	3	—	2	2	2	2	2	1	1	3	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	3	—	2	1	2	1	2	2	1	1	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	3	2	3	2	2	2	3	2	1	2	3	2	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
2	3	1	3	2	3	2	3	2	1	2	3	2	3	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
—	—	—	—	—	—	—	—	—	—	1	1	—	—	1	—	—	—	1	—	—	—	—	1	—	—	2	1	—	2	2	1	1	1	1	2	1	2	—	1	2	2	2	—
1	3	—	2	1	1	1	2	1	1	1	2	2	2	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1	2	1	3	3	2	1	1	3	2	3	3	3	2	2	3	1	3	3	2	1	2	2	3	1	2	1	3	2	1	—	1	3	3	2	2	2	2	1	3	2	2
—	—	—	—	—	—	—	—	—	—	1	—	—	—	1	—	—	—	—	—	1	—	—	—	1	—	—	—	1	—	—	—	—	—	—	—	—	—	—	—	—	—
1	1	—	2	2	1	—	1	1	1	—	1	2	1	1	3	—	2	2	1	1	2	1	3	1	2	1	2	2	1	—	—	2	2	2	2	1	1	1	2	2	1
1	—	1	2	2	1	1	1	1	3	2	2	1	1	1	—	2	2	2	—	—	1	2	1	3	—	2	1	1	—	—	1	2	1	1	1	1	1	—	2	1	

genomic expression libraries using a polyclonal IgG preparation and not a single ligand as a selective agent. The display of all genomically encoded peptides provides an effective means of selection independent of gene annotation and reading frame, as shown here with the identification of 14 immunoreactive peptides encoded in putative novel short ORFs.

The use of human antibodies exhibiting a high titer of antibody against *S. aureus* allowed the selection of proteins, which are expressed in vivo during an infection and are able to induce an immune response, thereby revealing new possible candidate vaccine peptides against *S. aureus*. However, note that some protective proteins might be nonimmunogenic or immunore-

TABLE 4. Selected novel putative ORFs

ORF[a]	No.[b]	Result of[c]:			Genome position[d] (nucleotides)	Orientation[e]	ORF length (amino acids)	Selected clone region (nucleotides)	Opposite ORF[f]	Name of opposite ORF product[f]
		Peptide ELISA	RT-PCR	NB						
PSAN1	6	ND	+	+	1553–1443	−	37	1535–1464	SAA0002	Tetracycline resistance protein
SAN1	9	N: 5/5	+	ND	11772–11671	−	34	11751–11708	SA0008	Histidine ammonia lyase
SAN2	8	N: 4/5	+	ND	135574–135699	+	42	135601–135660	SA0120	Bacterial regulatory proteins, gntR
SAN3	9	ND	+	ND	301822–301718	−	35	301816–301743	SA0263	Peptidoglycan hydrolase
SAN4	69	N: 4/5	+	ND	382929–382792	−	46	382878–382808	SA0379	phi PVL ORF 15 and 16 homologue
SAN5	7	ND	−	ND	1038052–1037975	−	26	1038085–1038018	SA1028	Hypothetical protein
SAN6	21	ND	+	+	1616061–1616270	+	70	1616109–1616161	SA1579	Conserved hypothetical protein
SAN7	86	P: 36/42 N: 29/32	+	+	1858035–1858460	+	142	1858134–1858159	SA1806	LPXTG-motif cell wall protein
SAN8	5	ND	+	ND	2122207–2122449	+	81	2122291–2122377	SA2055	*rsbW* protein
SAN9	5	ND	+	+	2221258–2221758	+	167	2221486–2221680	SA2150	*mrp* protein
SAN10	256	P: 34/42 N: 26/32	+	+	2276258–2276142	−	39	2276261–2494441	SA2194	Hyaluronate lyase
SAN11	8	N: 3/5	−	ND	2290161–2290319	+	53	2290173–2290241	SA2208	tRNA pseudouridine synthase A
SAN12	4	ND	+	ND	2723814–2723680	−	45	2723822–2723753	SA2661	Hypothetical protein
SAN13	3	N: 3/5	−	ND	2728321–2728259	−	21	2728315–2728266	SA2664	Mannose-6-phosphate isomerase

[a] Consecutive numbering of novel ORFs, SAN, *S. aureus* New; prefix P, localization on plasmid of *S. aureus* COL strain.

[b] Number of selected clones located in the respective putative ORF.

[c] Peptide ELISA results show reactivity with patient sera (P) or healthy-subject high-titer sera (N) (as described in reference 4). Expression analysis by RT-PCR and Northern blotting (NB) were also carried out. ND, not determined.

[d] Position of the putative ORFs on the genome according to CMR database (20).

[e] Orientation of the putative ORFs on the plus strand (+) or negative strand (−) of the genome.

[f] The opposite ORF and its name are the ORF number and name of the gene that is located in antisense direction to the putative novel ORF.
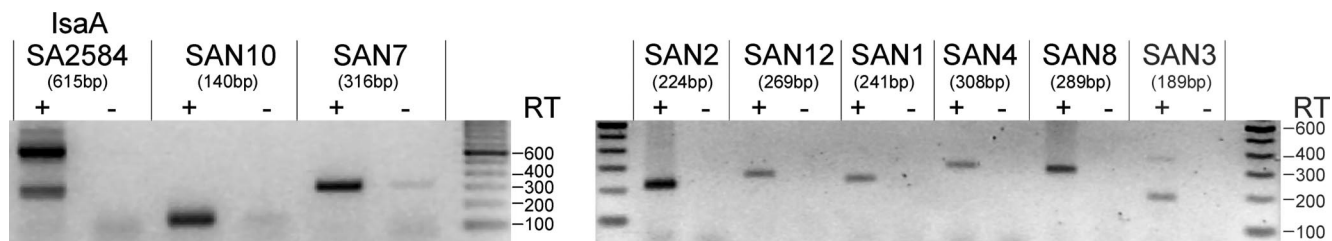
FIG. 3. Transcriptional analysis of putative short ORFs. In vivo transcription of putative novel ORFs was determined by RT-PCR. RNA was extracted from *S. aureus* COL. RT-PCR with specific primers for the indicated novel ORFs and the IsaA gene as a control was carried out. The expected length of the RT-PCR products is shown in parentheses. Lanes: −, controls without RT, followed by PCR; +, lanes subjected to RT-PCR.

cessive in *S. aureus* infections and may be only immunogenic upon massive encounter as recombinant proteins and therefore will be missed by our approach.

Although most of the identified peptides reacted with high-titer and healthy-patient sera to various extents, some alterations for individual peptides could be seen. Antibodies missing from sera of acute-phase patients can be considered especially important, since it can be speculated that those provide protection in the healthy individuals. It is an important task to identify these missing antibodies and their corresponding epitopes for the design of protective or preventive vaccination. However, it is beyond the scope of the present work. Notably, for some genes a cytoplasmic rather than a surface localization was expected from the function they were assigned to. However, even peptides derived from these genes react very strongly with a number of different sera. This could indicate that the localization of the respective proteins is not restricted to the cytoplasm but is also accessible to an immune attack (compare, e.g., reference 16), or they act highly immunogenic after destruction of the bacterial cell, leading to the release of cytoplasmic proteins and subsequent raising of specific antibodies. However, no highly abundant cytoplasmic proteins (e.g., ribosomal proteins or EF-Tu) were identified, making this interpretation less likely.

Surprisingly, a number of identified clones mapped to orientations opposite from those of known genes. Reactivity with serum and transcriptional activity in vivo was confirmed, therefore raising the possibility for the existence of novel putative short ORFs. Final proof would be the isolation of the corresponding proteins; these proteins are potential novel targets for vaccine development. However, it should be noted that positive serum reactivity could also arise from cross-reacting antibodies. On the other hand, it was recently observed that antisense transcription of bacterial and even mammalian genomes is a general phenomenon (24, 28), allowing the assumption that some of these antisense transcripts are translated in vivo. The concept of nontraditional or "hidden" antigens is known for T-cell epitopes (15) but has to our knowledge never been demonstrated to this extent for B-cell epitopes. Moreover, the evidence provided here for the existence of antisense transcripts to known genes could also indicate a regulatory function on the RNA level, as it was shown that antisense transcripts very efficiently turn down the expression of the corresponding genes (11). Interestingly, some of the putative short ORFs lie opposite to genes involved in virulence, like *tet* (which codes for tetracycline resistance protein), *mrp* (which codes for methicillin resistance protein), or *hysA* (which codes for hyaluronate lyase) (Table 4).

Finally we wanted to directly compare the expressibility of the identified clones in an in vivo-based system. About 55% of the identified clones could not be expressed in an FhuA-based surface display system in *E. coli*. Besides the possibility that toxic fragments interfere with growth, it is known that membrane permeation of certain protein domains is a challenge due to the high selectivity of the outer membrane translocase (1). Besides for bacterial surface display, membrane perme-
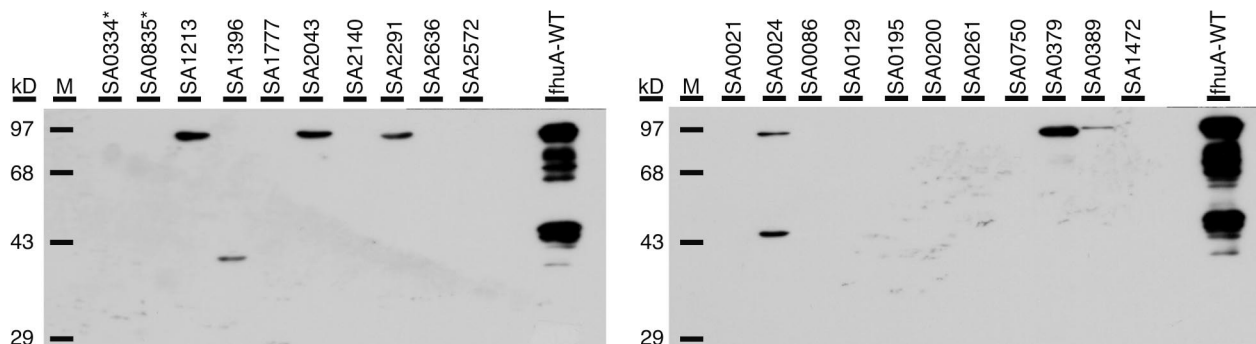

FIG. 4. In vivo expression analysis of in vitro-selected peptides. Assessment of in vivo expression by inserting 19 clones identified by in vitro selection into loop 5 of the *fhuA* gene. Recombinant *E. coli* was induced for *fhuA* expression, and extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with antibodies against FhuA. As controls, wild-type *fhuA* (positive control) and two clones (SA0835 and SA0334, indicated by an asterisk) containing a stop codon in the library fragment (negative controls), respectively, are shown.

ation is also required for (nonlytic) phage display (e.g., M13 based) as well as yeast display; our observation can therefore probably be generalized also to these systems, although the extent to which the generalization applies might vary. Interestingly, a major class of peptides that cannot be displayed belongs to membrane-associated transporter proteins (compare Fig. 4 and Table 1).

Taken together, our report provides evidence that in vitro protein selection allows the rapid and comprehensive identification of immunodominant peptides from genomic expression libraries and therefore to extensively map the human antibody response against a particular pathogen. Moreover, individual humoral responses between healthy individuals or patients can be compared on a large scale when performing screens with only one serum sample.

Furthermore, the identified peptides are excellent vaccine candidates. Due to the specific mapping of immunoreactive regions within a protein, it spares the often-difficult expression of the corresponding full-length recombinant protein by providing the possibility of using defined synthetic peptides for testing and formulation of vaccine preparations. These advantages suggest in vitro protein selection as a generally applicable approach for characterization of the humoral immune response and therefore aiding the identification of active or passive vaccine candidates.

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