INFECTION AND IMMUNITY, Nov. 2002, p. 6499–6503 Vol. 70, No. 11
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.11.6499–6503.2002

Multiparameter Selection of *Helicobacter pylori* Antigens Identifies Two Novel Antigens with High Protective Efficacy

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Received 1 April 2002/Returned for modification 28 June 2002/Accepted 22 July 2002

A multiparameter selection of *Helicobacter pylori* antigens for vaccine development identified 15 candidates, 6 of which are known protective antigens. Two novel antigens with low homology to other organisms (HP0231 and HP0410) were overexpressed and purified with high yields. Both confer protective immunity in the mouse *Helicobacter* infection model.

The gram-negative bacterium *Helicobacter pylori* is a widespread human pathogen that can cause gastritis, gastric and duodenal ulcers, and gastric cancer. In various preclinical animal models, vaccination has been shown to protect against a *Helicobacter* challenge infection (7). Most of the vaccines that have been tested contain only one or two antigens, but the results of recent studies suggest that combining several protective antigens can substantially increase vaccine efficacy (11, 22, 32). The two sequenced *H. pylori* genomes contain some 1,600 genes (2, 36), and appropriate parameters are needed to select a practical number of novel antigen candidates. In one study (10), more than 400 putative membrane- or surface-associated antigens were overexpressed, and about 100 of these could be obtained in sufficient yield and purity. When tested in the mouse *Helicobacter* infection model, 10 antigens were found to be protective, several of which had previously been identified by empirical approaches (10), suggesting that antigens can be identified in silico, although putative surface localization selection is a rather poor predictive parameter.

The results of studies with mice suggest that $CD4^+$ T cells are essential for protection against an *H. pylori* infection, while both $CD8^+$ T cells and antibodies appear to be dispensable (4, 9). Several parameters have been suggested as predictive indicators for the ability of a given antigen to induce potent $CD4^+$ T-cell responses, but in most cases, little experimental data exist to directly support such assumptions for *H. pylori* proteins.

T-cell responses are dose dependent (37), suggesting that abundant *H. pylori* proteins may be appropriate antigen candidates. Abundant proteins in *H. pylori* in vitro cultures have recently been identified by proteome analysis (16). However, the in vitro conditions are not likely to accurately reproduce the relevant in vivo situation despite the finding that for three specific genes, relative protein abundance in vitro parallels transcript levels in human stomach biopsy specimens (16, 29). Qualitative information about antigen expression in vivo can be obtained from immunoproteomics (13, 19, 23). Specific recognition of a *Helicobacter* antigen by sera from infected patients or animals suggests that this antigen is expressed in vivo and is accessible to the immune system.

The localization of a bacterial antigen can influence specific T-cell responses. In a number of pathogens, surface-exposed antigens are thought to be more efficient in inducing a cellular immune response than cytoplasmic antigens (17, 34). *H. pylori* colonizes the mucous layer and the apical side of gastric epithelia cells, whereas $CD4^+$ T cells that mediate protection reside in the mucosa. Secreted *Helicobacter* proteins and surface-associated proteins that are sequestered by vesicle budding are more likely to reach antigen-presenting cells in the mucosa for T-cell restimulation, as previously demonstrated for the best-characterized protective antigen urease (21). Indeed, the majority of known protective *Helicobacter* antigens are apparently surface exposed or secreted (35), and this property has been used with some success to predict novel antigens (10). Sixty-four putative surface-exposed proteins have been theoretically predicted for *H. pylori* (1). In addition, selective labeling followed by proteome analysis revealed 18 surface-associated proteins (30), and analysis of culture supernatants revealed 23 secreted proteins (5).

Isolates of *H. pylori* are genetically diverse (3, 31), and vaccines should preferably contain antigens that are highly conserved among different strains. The complete genome sequences of two independent strains and genetic information about various specific loci in multiple strains provide the necessary information to select conserved antigens.

The binding affinities of peptides to major histocompatibility complex class II molecules on antigen-presenting cells can be predicted on the basis of empirical data sets containing known T-cell epitopes (6, 24). Proteins that contain peptides with high theoretical T-cell epitope scores are likely to induce potent $CD4^+$ T-cell responses.

As most of the various selection parameters for protective *H. pylori* antigens are rather tentative, we combined them to select potential antigen candidates, assuming that most of the criteria have at least some relevance. At least 59 antigens are recognized by *H. pylori*-infected patients (13, 19, 23), and 48 of these antigens have a staining intensity that is higher than an arbitrary cutoff equivalent to 0.1% of the total staining intensity (16) (Table 1). Among the 48 seroreactive and abundant antigens, 15 appear to be secreted or surface associated (1, 5, 30),

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TABLE 1. Multiparameter selection of *H. pylori* antigens

Antigen	Gene[a]	Seroreactivity[b,c]	Staining intensity[c,d]	Surface localization[c,e]	Distribution in 15 isolates[c,f]	Predicted T-cell epitopes[c,g]	Homology to other organisms[h]	Protection
HP0010	*groEL*	+	5,596	Surf.	All genomes	5	Strong (1.6e − 193)	Yes (11)
HP0011	*groES*	+	1,477	ND	NA	NA	NA	NA
HP0027	*icd*	+	683	ND	NA	NA	NA	NA
HP0072	*ureB*	+	3,544	Surf.	All genomes	3	Strong (6.4e − 158)	Yes (25)
HP0073	*ureA*	+	1,774	Surf.	All genomes	2	Strong (3e − 199)	Yes (25)
HP0109	*dnaK*	+	177	Surf.	All genomes	8	Strong (4.9e − 57)	NA
HP0115	*flaB*	+	<100	ND	NA	NA	NA	NA
HP0153	*recA*	+	209	ND	NA	NA	NA	NA
HP0154	*eno*	+	214	ND	NA	NA	NA	NA
HP0175	cell binding factor 2 gene	+	655	Sec., Surf.	All genomes	3	Strong (9.3e − 31)	NA
HP0177	*efp*	+	340	ND	NA	NA	NA	NA
HP0192	*frdA*	+	264	ND	NA	NA	NA	NA
HP0231	Hypo. ORF	+	531	Sec., Surf.	All genomes	3	Weak (1.8e − 06)	Yes (this study)
HP0243	*napA*	+	673	Surf.	All genomes	1	Strong (3.5e − 22)	Yes (33)
HP0264	*clpB*	+	241	ND	NA	NA	NA	NA
HP0305	Hypo. ORF	+	320	ND	NA	NA	NA	NA
HP0318	Cons. hypo. ORF	+	227	ND	NA	NA	NA	NA
HP0371	*fabE*	+	340	ND	NA	NA	NA	NA
HP0400	*lytB*	+	130	ND	NA	NA	NA	NA
HP0410	*hpaA* homologue	+	415	Surf.	All genomes	4	None	Yes[i] (this study)
HP0512	*glnA*	+	397	ND	NA	NA	NA	NA
HP0522	*cag3*	+	93	ND	NA	NA	NA	NA
HP0537	*cag16*	+	76	ND	NA	NA	NA	NA
HP0547	*cag26*	+	1,194	Sec.	11 of 15	6	Weak (0.00012)	Yes (22)
HP0589	Ferredoxin oxidoreductase/gene	+	263	ND	NA	NA	NA	NA
HP0599	*hylB*	+	302	ND	NA	NA	NA	NA
HP0601	*flaA*	+	450	Surf.	All genomes	3	Strong (4.7e − 91)	NA
HP0649	*aspA*	+	340	ND	NA	NA	NA	NA
HP0691	*yxjD*	+	237	ND	NA	NA	NA	NA
HP0752	*fliD*	+	230	ND	NA	NA	NA	NA
HP0779	*acnB*	+	843	ND	NA	NA	NA	NA
HP0794	*clpP*	+	237	ND	NA	NA	NA	NA
HP0795	*tig*	+	450	ND	NA	NA	NA	NA
HP0829	*quaB*	+	212	ND	NA	NA	NA	NA
HP0875	Catalase gene	+	1,021	Surf.	All genomes	2	Strong (9.4e − 157)	Yes (28)
HP0900	*hypB*	+	341	ND	NA	NA	NA	NA
HP0912	*omp20*	+	150	Surf.	NA	NA	NA	NA
HP1018	Hypo. ORF	+	150	ND	NA	NA	NA	NA
HP1019	*htrA*	+	603	Sec., Surf.	All genomes	4	Strong (2.3e − 91)	NA
HP1037	*pepO*	+	213	ND	NA	NA	NA	NA
HP1098	Cons. hypo. secreted ORF	+	646	Surf.	All genomes	6	Strong (1.7e − 36)	NA
HP1110	Pyruvate ferredoxin oxidoreductase gene	+	250	ND	NA	NA	NA	NA
HP1125	*omp18*	+	900	Surf.	All genomes	1	Strong (1.3e − 23)	NA
HP1132	*atpD*	+	171	ND	NA	NA	NA	NA
HP1134	*atpA*	+	162	ND	NA	NA	NA	NA
HP1152	*ffh*	+	124	ND	NA	NA	NA	NA
HP1199	*rpl7/l12*	+	915	ND	NA	NA	NA	NA
HP1201	*rlpA*	+	398	ND	NA	NA	NA	NA
HP1205	*tufB*	+	727	ND	NA	NA	NA	NA
HP1285	Cons. hypo. ORF	+	59	ND	NA	NA	NA	NA
HP1293	*rpoA*	+	273	ND	NA	NA	NA	NA
HP1302	*recA*	+	603	ND	NA	NA	NA	NA
HP1307	*rplE*	+	397	ND	NA	NA	NA	NA
HP1350	Protease gene	+	154	ND	NA	NA	NA	NA
HP1555	*tsf*	+	251	ND	NA	NA	NA	NA
HP1563	*tsaA*	+	2,122	ND	NA	NA	NA	NA
HP1564	*omp*	+	434	Surf.	All genomes	3	Strong (5.4e − 58)	NA
HP1582	*pdxJ*	+	355	ND	NA	NA	NA	NA

[a] Abbreviations: Hypo., hypothetical; ORF, open reading frame; Cons., conserved.
[b] Data from references 13, 19, and 23.
[c] The particular criteria matching proteins are boxed.
[d] Arbitrary units as determined from spot intensities of *H. pylori* two-dimensional gels using the TOPSPOT program (16). An arbitrary threshold of 175 equivalent to 0.1% of the total staining intensity was used.
[e] Data from references 1, 5, 15, 26, 27, and 30. Abbreviations: Sec., secreted; Surf., surface; ND, not detected.
[f] Presence as determined by DNA-microarray hybridization (31). NA, not analyzed.
[g] Murine *H-2*^{d} major histocompatibility complex class II-restricted T-cell epitopes were predicted by comparing the amino acid sequence to those of known T-cell epitopes (20); the number of nonoverlapping epitopes with a predicted score equal or higher than that of the well-defined ovalbumin T-cell epitope (amino acids 323 to 339) are given. NA, not analyzed.
[h] Homology (*P* value) for the closest non-*Helicobacter* homologue in the Comprehensive Microbial Database (at http://www.tigr.org). A *P* value below 1.0×10^{-20} is called strong homology. A *P* value of 0.001 to 1.0×10^{-20} is called weak homology. NA, not analyzed.
[i] If present in challenge strain.

TABLE 2. Oligonucleotide primers used

Antigen	Primer direction	Sequence (5′ to 3′)[a]
HP0231	Forward	TTAGGAGTTCATATGATATTAAGAGC
	Reverse	GCGATATCGGATCCGTCGACTAATGATGATGATGATGATGTGCCTTATAATGGTATAAGAAA
HP0410	Forward	GAAAGGAATCATATGAAAAAAGGT
	Reverse	GCGATATCGGATCCGTCGACTAATGATGATGATGATGATGCTTTCGTTTTTTCATTTCAC
HP1098	Forward	AGGAGATACCATATGTTAGAAAATGTC
	Reverse	GCGATATCGGATCCGTCGACTAATGATGATGATGATGATGAACTTTGATCTTAAGCTGCTT

[a] Underlined sequence regions indicate *Nde*I and *Bam*HI recognition sites used for cloning.

and almost all are present in all 15 isolates analyzed (except Cag26) and contain at least one putative T-cell epitope (Table 1). Interestingly, this set of 15 potential *Helicobacter* antigens contains six proteins that have already been shown to be highly protective in the mouse infection model, supporting the utility of our selection strategy. Immunization trials with a large set of antigen candidates will be required to validate each of the presently used and other potential selection parameters to further improve the approach. Moreover, the different data sets are still incomplete, and there are probably more antigen candidates. However, the already achieved high selection success rate motivated us to further characterize some of the new candidates.

To identify novel attractive antigens with minimal cross-reactivity, we selected three candidates with weak homology to other organisms (Table 1) (homology derived from the Comprehensive Microbial Database at http://www.tigr.org): the hypothetical protein HP0231, the putative neuraminyllactose-binding hemagglutinin HpaA homologue HP0410, and the hypothetical secreted protein HP1098 that was later found to have a homologue with high similarity in *Magnetococcus* sp. strain MC-1. The corresponding genes were PCR amplified from chromosomal DNA from strain P76 (12) using the primers shown in Table 2, and cloned into pET15b (Novagen). The His_6-tagged proteins were overexpressed in *Escherichia coli* BL21(DE3) and purified by cobalt affinity chromatography. HP0231 and HP0410 could be recovered from inclusion bodies of induced *E. coli* cultures at high purity and yields (Fig. 1). Interestingly, a soluble form of HP0410 was also recovered from culture supernatants. The soluble form has a somewhat lower apparent molecular weight compared to that of the insoluble form and may have been processed by signal peptide cleavage. In contrast to HP0231 and HP0410, HP1098 was only weakly expressed in *E. coli* even under inducing conditions and was therefore not investigated further.

The HP0231 and HP0410 antigens were individually tested for protective efficacy in groups of 5 to 10 female 6- to 8-week-old female BALB/c mice with specific-pathogen-free health status using four orogastric administrations (days 0, 21, 28, and 35) of 100 μg of purified protein in 100 μl of phosphate-buffered saline (PBS) containing 10 μg of the mucosal adjuvant cholera toxin. Four to six weeks after the last immunization, the mice were challenged with one or three orogastric doses of 2×10^8 to 5×10^8 CFU of the mouse-adapted *H. pylori* strain P76; four to six weeks later, the mice were sacrificed under anesthesia, and *H. pylori* stomach load and urease activity were determined as described previously (12). Compared to the sham-immunized control group, mice that had received HP0231 or HP0410 were protected against an *H. pylori* challenge infection (Fig. 2), with levels of protection (median CFU of 8% compared to that for the controls) equivalent to previous results for the best known antigens (8, 11, 14, 18, 25, 28, 32, 35) and approximating those of an immunization control group that had received four doses of 500 μg of P76 lysate, which is generally considered the gold standard for *Helicobacter* immunization (median CFU of 4% compared to that for the controls) (Fig. 2). There was no significant difference in protective efficacy between soluble and insoluble forms of HP0410 (data not shown). The protective effect of immunization with HP0231 or HP0410 was also evident from determinations of urease activity in the stomach samples ($P > 0.0001$ [*t* test] for both proteins; data not shown). Immunization with HP0231, but not with HP0410, induced specific serum antibodies that could be detected both by Western blotting and enzyme-linked immunosorbent assay ($P > 0.005$ [*t* test] compared to sham-immunized control group; data not shown). This suggests that serum antibody responses do not correlate with protective efficacy, which is in agreement with the results of previous studies (4, 9).

In conclusion, a combination of theoretical and experimental selection parameters predicts protective *H. pylori* antigens with a success rate (at least 8 of 15 predicted antigens) that is

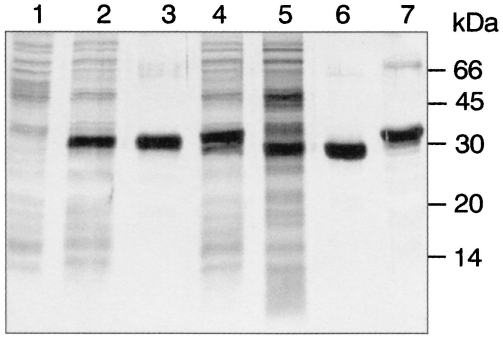

FIG. 1. Expression and purification of recombinant *H. pylori* antigens as analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and silver staining. Lane 1, *E. coli* BL21; lane 2, *E. coli* BL21 expressing HP0231; lane 3, purified HP0231 from inclusion bodies; lane 4, *E. coli* BL21 expressing HP0410; lane 5, supernatant of *E. coli* BL21 expressing HP0410; lane 6, purified HP0410 from supernatant; lane 7, purified HP0410 from inclusion bodies. The positions of molecular mass markers (in kilodaltons) are indicated to the right of the gel.

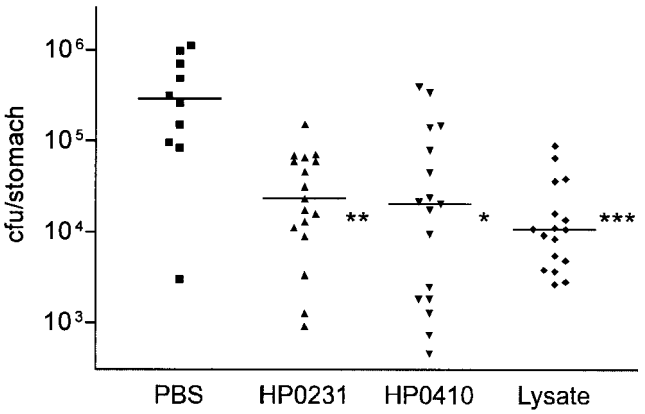

FIG. 2. Murine *H. pylori* stomach loads after oral immunization and an *H. pylori* P76 challenge. Combined results from two independent vaccination experiments are shown (a total of 10 mice tested for PBS; a total of 17 mice tested (each) for HP0231, HP0410, and lysate). The horizontal lines represent medians. Statistically significant differences compared to the values for the sham-immunized control group (PBS) were analyzed with the *t* test (*, $P < 0.005$; **, $P < 0.001$; ***, $P < 0.0005$).

superior to previous attempts. Two novel antigens identified in this study have protective efficacies similar to those of the best previously known antigens and, unlike most other protective antigens, are highly specific for *Helicobacter*. Further studies will help to validate individual selection parameters for further improvement of the combination approach.

We thank T. Aebischer for helpful discussions.

This study was supported in part by the Deutsche Forschungsgemeinschaft (Me 756/6-1).

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Editor: E. I. Tuomanen