

OMICS Techniques and Identification of Pathogen Virulence Genes Application to the Analysis of Respiratory Pathogens

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Abstract

The advent of genomics should have facilitated the identification of microbial virulence factors, a key objective for vaccine design, especially for live attenuated vaccines. It is generally assumed that when the bacterial pathogen infects the host it expresses a set of genes, a number of them being virulence factors. However, up to now, although several *Omics* methods have been applied to identify virulence genes, i.e., DNA microarrays, In Vivo Expression Technology (IVET), Signature-Tagged Mutagenesis (STM), Differential Fluorescence Induction (DFI), etc., the results are quite meager. Among the genes identified by these techniques there are many related to cellular stress, basal metabolism, etc., which cannot be directly involved in virulence, or at least cannot be considered useful candidates to be deleted for designing a vaccine. Among the genes disclosed by these methodologies there are a number annotated as being hypothetical or unknown proteins. As these ORFs can hide some true virulence factors, we have selected all of these hypothetical proteins from several respiratory diseases and predicted their biological functions by a careful and in-depth analysis of each one. Although some of the re-annotations match with functions that can be related to microbial virulence, it can be concluded that identification of virulence factors remains elusive.

Introduction

Vaccination is the method of choice to fight microbial pathogens and presents the best cost/benefit ratio among current clinical and pharmaceutical practices. The advent of genomics and high throughput methods should facilitate the identification of potential virulence factors and main antigens of a pathogen, through the so-called reverse vaccinology (Pizza et al., 2000; Grandi, 2001; Maione et al., 2005; Scarselli et al., 2005; Rodriguez-Ortega et al., 2006). One of the most interesting strategies for vaccine design is based on live attenuated pathogens and requires the previous identification of those genes involved in pathogenicity and

virulence. However, in general, only a very small fraction of the pathogen proteins, sometimes only one (i.e., a toxin), appears to be involved in virulence. The best situation would be that in which genome sequences for both exist, the harmless one and one or more virulent forms, and that both strains present few differences in their genome/proteome. In these cases, the identification of putative virulence factors could be straightforward, but this is not the typical point of departure. Therefore, even in the genomics age, the identification of virulence factors remains a very difficult task. In practice, and for vaccine purposes, that a gene is

involved in pathogenicity and virulence, or in any other function, can only be demonstrated upon its deletion, sometimes followed by a complementation assay (Pich et al., 2006).

Sometimes the gene/protein may be a true virulence factor, but its deletion does not lead to an effective vaccine strain because the microorganism has alternative pathways or proteins to perform the function. This is the case of the iron-acquisition systems in *Actinobacillus pleuropneumoniae*, whose Transferrin-binding protein 1 we identified and cloned (Daban et al., 1996). Upon deleting the Tbp1 gene, the strain remains as virulent as the wild-type strain. Another problem for live attenuated vaccines, as also reported for *A. pleuropneumoniae*, is that some true virulence factors such as haemolysins cannot be deleted because, although the strain becomes non-virulent, it also loses its protective immunogenicity. In this case, we have overcome the problem and designed a protective live-strain predicting, and further deleting, one of the putative transmembranes that forms the lytic pore but preserving the rest of the protein structure, which, since it retains the native conformation, is immunogenic and protective (Bru et al., 2002).

In general, in order to obtain a protective vaccine strain, it is easier to identify virulence genes from viruses rather than from bacteria, since even large viruses present fewer functional classes (Rebordosa et al., 1994).

Also elusive is the determination of which of the surface proteins (the so-called Surfome, since Rodriguez-Ortega et al., 2006) can be useful for reverse vaccinology. That is, a good target to be *E. coli*-expressed and able to elicit protective immunity as a recombinant subunit vaccine. We have recently reported a simple starting rule that suggests that researchers should discard those proteins that share protein epitopes with a host protein, as they could lead to an autoimmune disease and thus the host would not elicit antibodies (Amela et al., 2007). Work is in progress to computationally predict, from the protein sequences, the actual main protective antigens among the hundreds of proteins from the Surfome.

For recombinant vaccine design the final objective is to identify a pathogen's targets in order to decide among several different strategies, i.e., a gene knock-out of a virulence factor; recombinant expression of an immunogenic protective protein, etc. And in the case of some pathogens like

Mycobacterium tuberculosis, a species-specific metabolic enzyme, which is a putative target for a small-molecule drug, can be included. From the genomics analysis, any gene that is present in the pathogenic strain and absent in a non-virulent one can be considered to be a virulence-pathogenicity gene. They can be directly responsible for pathological damage during infection (for example, a toxin), be involved in the interaction or colonisation of the host cells or be related to the acquisition of molecules and metabolites by the pathogen (i.e. iron), or enable the pathogen to evade the host immune system (Strauss and Falkow, 1997; Wassenaar and Gaastra, 2001; Marra, 2003).

Among the strategies to disclose virulence factors are DNA arrays, differential proteomics' and others that try to find bacterial promoters activated when the microorganism infects the host, for example, In Vivo Expression Technology (IVET) (Mahan et al., 1993), Signature-Tagged Mutagenesis (STM) (Hensel et al., 1995), Differential Fluorescence Induction (DFI) (Valdivia and Falkow, 1997), Selective Capture of Transcribed Sequences (SCOTS) (Baltes and Gerlach, 2004), etc. The question is: Did these methods find any of the desired targets? (In this work we analyse a number of results reported elsewhere using these techniques for bacterial pathogens related to respiratory diseases, with a significant number of sequenced pathogen genomes.) A number of genes found in the experiments by IVET, STM, DFI, SCOTS and DNA microarrays correspond to hypothetical or unknown proteins. These proteins, and especially those species-specific, can code for pathogenicity and virulence factors. In previous works we have identified some *Mycoplasma* virulence factors among hypothetical proteins (Pich et al., 2006; Burgos et al., 2006). Therefore, we have predicted the biological function of these hypothetical proteins by means of a careful bioinformatics analysis of all of them.

Pathogens Analysed

For our analysis, some respiratory microbial pathogens have been chosen of which IVET, STM, DFI, SCOTS and DNA microarrays assays have been reported elsewhere. A Table in Supplementary Material shows the list of the different protein sequences disclosed by these techniques upon host infection by a respiratory pathogen. Respiratory pathogens have been chosen for the analysis for the following reasons: (a) they are important pathogens, both for humans and livestock, (b) it is possible that, its niche being the respiratory system, they may share infection and

pathogenicity mechanisms, even host targets, and (c) there are enough sequenced genomes and reported experimental analyses applying *Omic*s techniques.

Data analysed and discussed in the present work are from the following microbial pathogens: *Actinobacillus pleuropneumoniae* (Fuller et al., 2000b; Sheehan et al., 2003; Baltes and Gerlach, 2004; Baltes et al., 2003; Moser et al., 2004; Hodgetts et al., 2004; Jenner and Young, 2005; Jacobsen et al., 2005a,b,c; Deslandes et al., 2007; Wagner and 2007; Hedegaard et al., 2007); *Pasteurella multocida* (Fuller Mullks et al., 2000a; Hunt et al., 2001; Paustian et al., 2002; Harper et al., 2003; Boucher et al., 2005); *Bordetella avium* (Hot et al., 2003; Spears et al., 2003); *Staphylococcus aureus* (Palmqvist et al., 2002; Benton et al., 2004); *Haemophilus influenzae* (Herbert et al., 2002; Gilsdorf et al., 2004); *Legionella pneumophila* (Edelstein et al., 1999; Polesky et al., 2001); *Pseudomonas aeruginosa* (Lehoux et al., 2002; Wang et al., 1996; Woods, 2004); *Streptococcus pneumoniae* (Marra et al., 2002; Orihuela et al., 2004); *Chlamydia pneumoniae* (Mahony, 2002); *Yersinia pseudotuberculosis* (Karlyshev et al., 2001).

All protein sequences were also checked for their inclusion in virulence factors databases such as BacBix and PRINTS virulence factors database (Paine and Flower, 2002), which can be found at the website:

<http://www.jenner.ac.uk/BacBix3/Welcomehomepage.htm>

and MannDB (Zhou et al., 2006, 2007), a microbial database of automated protein sequence analyses (<http://manndb.llnl.gov/>), which also contains a link that allows one to find the bacterial virulence factors website:

<http://mvirdb.llnl.gov/>.

Results and Discussion

Table 1 shows those genes identified in more than one microorganism by any one of the IVET, STM, DFI, SCOTS and DNA microarrays techniques (The complete list of 819 genes-proteins taken from the above-mentioned bibliography is showed as Supplementary Material in <http://bioinf.uab.es/JCSB>). An immediate conclusion made from Table 1 is that in rare cases is a gene disclosed by more than one technique. Worse, results using the same technique by different laboratories do not match either. It is the case of putative virulence genes from *Actinobacillus pleuropneumoniae* disclosed by STM and SCOTS (Sheehan et al., 2003; Baltes

and Gerlach, 2004). Still, many of the disclosed putative virulence genes by these techniques cannot be found in databases of microbial virulence factors, like BacBix (Paine and Flower, 2002), but a number of them (75 out of 819 genes) can be found in the more exhaustive MannDB database (Zhou et al., 2006, 2007), which includes most bacterial genes. Many of these 75 factors are related to virulence in a broad sense (iron metabolism, etc.) although a number of them are actually related, i.e., five toxins, six haemolysins and 10 from LPS biosynthesis.

Also remarkable is that *Omic*s techniques usually fail in identifying main virulence factors previously known from experimental work. For example, in the case of *A. pleuropneumoniae*, only SCOTS (Sheehan et al., 2003; Baltes and Gerlach, 2004) disclose haemolysin ApxIV, which is a real host-pathogen-interacting haemolysin, and an iron gene which is also a gene necessary for iron capturing. However, as indicated in the Introduction, neither of them correspond to virulence factors which could lead to an effective vaccine. The main virulence factors of *A. pleuropneumoniae* are the RTX haemolysin ApxI, and sometimes, the ApxII cytolysin (Jansen et al., 1995; Reimer et al., 1995; Piñol et al., 2002).

Many of the reported genes activated when the pathogen infects the host correspond to proteins related to stress and protein folding (heat-shock proteins, thioredoxin, disulphide isomerases, etc.). This result should be expected, as the pathogen is under the pressure of the defence countermeasures activated by the host (which includes fever, nitrous oxide from macrophages, etc), and these are proteins that enable the survival of the pathogen under stress conditions. Also expressed are catalases in order to counteract free radicals produced by the host. Another common finding is the activation of basal metabolic genes, which could also be justified as above by the pathogen response to the host, by the pathogen cell-growth, etc. It is known that some pathogen metabolism enzymes can sometimes produce oxidative stress in the host cell and therefore they can be considered as true virulence factors. For example, in *Mycoplasma mycoides*, the enzyme α -glycerophosphate oxidase, which is involved in glycerol metabolism, produces H_2O_2 , which causes host-cell death. Nevertheless, although in a broad sense they could be considered as being virulence factors, it is unlikely that the deletion of such metabolism genes would lead to a live-attenuated vaccine, which is the main final objective of these techniques. However, some metabolism proteins could

TABLE 1: GENES SHARED BY DIFFERENT MICROORGANISMS												
ANNOTATION	MICROORGANISM											
	<i>Actinobacillus Pleuropneumoniae</i>	<i>Pasteurella multocida</i>	<i>Bordetella pertussis</i>	<i>Haemophilus influenzae</i>	<i>Pseudomonas aeruginosa</i>	<i>Streptococcus pneumoniae</i>	<i>Staphylococcus aureus</i>	<i>Bordetella avium</i>				
DETECTION TECHNIQUE												
DETECTION TECHNIQUE	SCOTS	STM	IVET	STM	DNA arrays	IVET	DNA arrays	STM	IVET	STM	DFI	STM
Purine biosynthesis				purF						purF		
ATP synthase F1 gamma chain		atpG		atpG						atpG (arrays)		
Filamentous hemagglutinin				lspA1 fhaB1	lspA1 fhaB1							lspA1 fhaB1
Haemolysin/secretion accessory protein				lspB fhaC	lspB fhaC							lspB fhaC
Accessory protein - Ton dependent transport of iron compounds		exbB		exbB								
Polynucleotide phosphorylase		pnp		pnp								
De novo purine biosynthesis				purN						purN		
Pyrimidine biosynthesis				pyrF						pyrF		
ATP synthase					AtpA					AtpA (arrays)		
Ferric uptake regulator		Fur			Fur				Fur			
Energy transducer		TonB			TonB							
Heat shock protein-molecular chaperone		DnaK			DnaK		DnaK			DnaK (arrays)		
Thiol:disulfide interchange	DsbA	DsbA			DsbA							
Chaperone protein					GroEL					GroEL		
Serine protease					htrA					htrA (arrays)		
ATP-dependent protease		Lon			Lon							
ABC-type Fe ³⁺ transport system, periplasmic component					FbpA						FbpA	
30 S ribosomal protein S1					S1					S1 (arrays)		
Hemoglobin-binding protein	hgbA	hgbA										
Outer membrane protein	pomA	pomA										
Recombination		recR								recR (arrays)		
ATP synthase		atpH								atpH (arrays)		
Chaperone protein		dnaJ					dnaJ					
Threonine dehydratase									ilvA	ilvA (arrays)		
GMP synthase	guaA	guaA										
Leucyl-tRNA synthetase	tRNA-leu	tRNA-leu										
Fructose-bisphosphate aldolase										fba (arrays)	fba	
Elongation factor Tu	tufA										tufA	
Lactate dehydrogenase	ldh										ldh	
Putative GTP pyrophosphokinase					relA					relA (arrays)		
Heat shock protein		htpG					htpG	htpG				
Outer membranes lipoprotein pcp precursor		pcp				pcp						
Spermidine/putrescine transport		potD								potD		

provide new targets for antimicrobial drug development.

Several especially interesting functional classes for microbial virulence and vaccine design are adhesins, pili or fimbrial-type surface structures, gliding genes, OMPs and transporters. Motility and adhesion are required for efficient invasion of host cells. Adhesins are usually true virulence factors, since they enable colonisation of the host and, therefore, are key candidates for subunit vaccines. Detection of these proteins by assays such as Western Blot is very useful for their consideration in subunit vaccine strategies. However, few of the reported genes shown in Table I correspond to these proteins. Related to both metabolism and adhesion is the fact that in many cases adhesion starts by a pilus-mediated phase which is followed by upregulation of ammonium, Cl^- and SO_4^{2-} transport, transferrin Fe^+ uptake, other ABC transporters, and aminoacid metabolism. Enzymes such as oxydoreductases can also play a role in adhesion and, in the case of Gram⁺ bacteria, glyceraldehyde-3-phosphate dehydrogenase can be found since they mediate communication with the host (Grifantini et al., 2002). This enzyme is also responsible for binding host mucose mucines in the case of *Mycoplasma genitalium* (Alvarez et al., 2003). In any case, especially with transporters, a number of such functions is listed in the reported works and a summary is included in Table 1.

Transporters are another remarkable functional class for pathogenicity. In principle, they could be virulence factors in the class generically classified as “acquisition” (Strauss and Falkow, 1997), but, as far as we know, there are no cases of live vaccines whose protective immunity is based on these proteins. For example, upon the identification and cloning of the Transferrin-binding protein 1 gene (Daban et al., 1996), the *Tbp1* gene from *A. pleuropneumoniae* was knocked out and it was found to be not protective at all, probably because this microorganism has more than one single system for uptaking key metabolites like iron.

The existence of a core of metabolically important genes that are usually highly expressed in most microorganisms has been reported (Carbone, 2006; Puigbo et al., 2007). There are about two-hundred genes, their main functional classes correspond to genes involved in processes such as replication, transcription and translation machineries, chaperones like GroEL and GroES, and several genes involved in the metabolism of biomolecules. A number of them is disclosed by the *Omic*s techniques analysed in this study, for example, genes such as *purF*, *pyrF*, *pnp*, *atpG*, *atpA*, *atpH*, *exbB*,

potD, etc. Therefore, it is not surprising that since this group of genes is essential in the maintenance of life in most species, they can also be found in infectious processes.

Among virulence factors are genes involved in lipopolysaccharide (LPS) biosynthesis. The O side-chain of LPS is an important factor in the virulence of a range of pathogens, as it can mediate resistance to complement-mediated and phagocyte killing, or they might also have a role in the survival of the pathogen in the host, as in the case of *Y. pestis* (Parkhill et al., 2001), in which they protect the bacterium from cationic peptides of the human intestine. A number of genes involved in LPS biosynthesis is identified by these techniques, specifically 28 out of 819 (see Supplementary Material).

Another functional class that in many cases can be considered virulence factors directly responsible for pathological damage during initial phases of infection and colonisation is proteases. The list of genes (see Supplementary Material) shows an important number of proteases.

An additional possibility is that a number of proteins identified by *Omic*s techniques and annotated as being involved in basal metabolism can be moonlighting proteins, presenting more than one function (Jeffery, 1999), and that second function is actually involved in virulence. There are some known examples: in addition to its role in the glycolysis pathway, the glyceraldehyde-3-phosphate dehydrogenase enzyme is also responsible for host adhesion and communication (Alvarez et al., 2003; Grifantini et al., 2002). In fact, this enzyme has been involved more times in functions other than its glycolytic role (Kim and Dang, 2005). Another example is the piruvate dehydrogenase- β -subunit, which, in *Mycoplasma pneumoniae*, interacting with the Tu elongation factor, acts as a fibronectin receptor (Dallo et al., 2002). Still, glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase or piruvate dehydrogenase from *Mycoplasma pneumoniae* are present in the cytoskeleton of the microorganism; therefore, they could be involved in virulence one way or another.

In conclusion, in few cases do results from *Omic*s techniques such as IVET, STM, DFI, SCOTS and DNA microarrays match with functions that can easily be related to virulence, at least in the sense that the corresponding genes could be deleted in order to obtain a live-attenuated-vaccine. Identification of such virulence factors remains quite elusive, however, and as a way to gain an understanding of

the exploration process of new therapeutic approaches, using *Omic*s techniques may be the inclusion of supplementary information both from the host and the pathogen.

The underlying reason is because in some cases microorganism virulence is greatly dependent on the host interaction; one example could be gas gangrene. *Clostridium* infection is particularly dangerous when infected tissue is injured, creating an anaerobic environment. In fact, this type of microorganisms would be considered as being saprophytes. Sometimes, these host-pathogen interactions are not easy to be established because they depend on external factors, for instance, an apparently innocuous practice like iron supplementation on diet intake does appear to increase susceptibility to malaria (Prentice, 2008).

In other words, without considering the host-pathogen relation and interaction type, it is impossible to obtain an overall view of the problem, and in some cases proposed therapeutic measures would even be counterproductive.

As another general aspect to design a protective vaccine, it is necessary to consider the type of virulence of the considered microorganism to understand a good vaccination strategy. In aggressive microorganisms, classical approaches to block toxins produced or their invasive behaviour, can be applied to construct a protective vaccine. But in little-aggressive microorganisms an alternative approach would be to reduce their capability to resist the host immune-system attacks instead of generating a defective microorganism.

Nowadays, high throughput techniques applied to vaccine design are greatly dependent on an expert who gives insight to those apparently inconclusive results in order to rescue a putative vaccine target from the long list of hypothetical candidates, if any exists.

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