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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 than 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 *Omics* 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; Gilsdorfet 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 *Omics* 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											
	Actinobacillus Pleuropneumoniae		*Pasteurella multocida*		*Bordetella pertussis*	*Haemopihlus influenzae*		*Pseudomonas aeruginosa*		*Streptococcus pneumoniae*	*Staphylococcus aureus*	*Bordetella avium*
	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 **fhaB**1
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 Fe3+ 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 $SO4^{2-}$ 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 *Omics* 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 *Omics* 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 *Omics* 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 *Omics* 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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References

1. Álvarez RA, Blaylock MW, Baseman JB (2003) Surface localized glyceraldehyde-3-phosphate dehydrogenase of *Mycoplasma genitalium* binds mucin. Mol Microbiol 48: 1417–1425. » CrossRef » Pubmed » Google Scholar

2. Amela I, Cedano J, Querol E (2007) Pathogen Proteins Eliciting Antibodies do not Share Epitopes with Host Proteins: A Bioinformatics Approach. PLOS One 2: e512. » CrossRef » Pubmed » Google Scholar

3. Baltes N, Gerlach GF (2004) Identification of Genes Transcribed by *Actinobacillus pleuropneumoniae* in Necrotic Porcine Lung Tissue by Using Selective Capture of Transcribed Sequences. Infect Immun 72: 6711-6716. » CrossRef » Pubmed » Google Scholar

4. Baltes N, Hennig-Pauka I, Jacobsen I, Gruber AD, Gerlach GF (2003) Identification of Dimethyl Sulfoxide Reductase in *Actinobacillus pleuropneumoniae* and Its Role in Infection. Infect Immun 73: 6784-6792. » CrossRef » Pubmed » Google Scholar

5. Benton BM, Zhang JP, Bond S, Pope C, Todd C, et al. (2004) Large-Scale Identification of Genes Required for Full Virulence of *Staphylococcus aureus*. J Bacteriol 186: 8478-8489. » CrossRef » Pubmed » Google Scholar

6. Boucher DJ, Adler B, Boyce JD (2005) The *Pasteurella multocida nrfE* gene is upregulated during infection and is essential for nitrite reduction but not for virulence. J Bacteriol 187: 2278-2285. » CrossRef » Pubmed » Google Scholar

7. Daban M, Medrano A, Querol E (1996) Cloning, sequencing and expression of the transferrin-binding protein 1 gene from *Actinobacillus pleuropneumoniae*. Biochem J 315: 257-264. » CrossRef » Pubmed

8. Dallo SF, Kannan TR, Blaylock MW, Baseman JB (2002) Elongation factor Tu and E1 beta subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in *Mycoplasma pneumoniae*. Mol Microbiol 46: 1041-1051. » CrossRef » Pubmed » Google Scholar

9. Deslandes V, Nash JH, Harel J, Coulton JW, Jacques M (2007) Transcriptional profiling of *Actinobacillus pleuropneumoniae* under iron-restricted conditions. BMC Genomics 13: 8-72. » CrossRef » Pubmed » Google Scholar

10. Fuller TE, Kennedy MJ, Lowery DE (2000a) Identification of Pasteurella multocida virulence genes in a septicemic mouse model using signature-tagged mutagenesis. Microb Pathog 29: 25-38. » CrossRef » Pubmed » Google Scholar

11. Fuller TE, Martin S, Teel JF, Alaniz GR, Kennedy MJ, et al. (2000b) Identification of *Actinobacillus*

pleuropneumoniae virulence genes using signature-tagged mutagenesis in a swine infection model. Microb Pathog 29: 39-51. » CrossRef » Pubmed » Google Scholar

12. Gilsdorf JR, Marrs CF, Foxman B (2004) *Haemophilus influenzae*: Genetic Variability and Natural Selection To Identify Virulence Factors. Infect Immun 72: 2457-2461. » CrossRef » Pubmed » Google Scholar
13. Gómez A, Cedano J, Hermoso A, Espadaler J, Piñol J, et al. (2008) Prediction of protein function improving sequence remote alignment search by a fuzzy logic algorithm. Protein J 27: 130-139 . » CrossRef » Pubmed » Google Scholar
14. Grandi G (2001) Antibacterial vaccine design using genomics and proteomics. Trends Biotechnol 19: 181-8. » CrossRef » Pubmed » Google Scholar
15. Grifantini R, Bartolini E, Muzzi A, Draghi M, Frigimelica E, et al. (2002) Previously unrecognized vaccine candidates against group B meningococcus identified by DNA microarrays. Nat Biotechnol 20: 914-21. » CrossRef » Pubmed » Google Scholar
16. Harper M, Boyce JD, Wilkie IW, Adler B (2003) Signature-Tagged Mutagenesis of *Pasteurella multocida* Identifies Mutants Displaying Differential Virulence Characteristics in Mice and Chickens. Infect Immun 71: 5440-5446. » CrossRef » Pubmed » Google Scholar
17. Hedegaard J, Skovgaard K, Mortensen S, Sørensen P, Jensen TK, et al. (2007) Molecular characterisation of the early response in pigs to experimental infection with *Actinobacillus pleuropneumoniae* using cDNA microarrays. Acta Vet Scand 49: 11. » CrossRef » Pubmed » Google Scholar
18. Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, et al. (1995) Simultaneous identification of bacterial virulence genes by negative selection. Science 269: 400-403. » CrossRef » Pubmed » Google Scholar
19. Herbert MA, Hayes S, Deadman ME, Tang CM, Hood DW, et al. (2002) Signature Tagged Mutagenesis of *Haemophilus influenzae* identifies genes required for in vivo survival. Microb Pathog33: 211-223.» CrossRef » Pubmed » Google Scholar
20. Hodgetts A, Bossé JT, Kroll JS, Langford PR (2004) Analysis of differential protein expression in *Actinobacillus pleuropneumoniae* by Surface Enhanced Laser Desorption Ionisation—ProteinChipTM (SELDI) technology Vet Microbiol 99: 215-225.» CrossRef » Pubmed » Google Scholar
21. Hunt ML, Boucher DJ, Boyce JD, Adler B (2001) In vivo-expreseed genes of *Pasteurella multocida*. Infect Immun 69: 3004-3012.» CrossRef » Pubmed » Google Scholar
22. Jacobsen I, Hennig PI, Baltes N, Trost M, Gerlach GF (2005a) Enzymes involved in anaerobic respiration appear to play a role in *Actinobacillus pleuropneumoniae* virulence. Infect Immun 73: 226-234. » CrossRef » Pubmed » Google Scholar
23. Jacobsen I, Gerstenberg J, Gruber AD, Bossé JT, Langford PR, et al. (2005b) Deletion of the ferric uptake regulator fur impairs the in vitro growth and virulence of *Actinobacillus pleuropneumoniae*. Infect Immun 73: 3740-3744. » CrossRef » Pubmed » Google Scholar
24. Jacobsen I, Meens J, Baltes N, Gerlach GF (2005) Differential expression of non-cytoplasmic Actinobacillus pleuropneumoniae proteins induced by addition of bronchoalveolar lavage fluid. Vet Microbiol 109: 245-256. » CrossRef » Pubmed » Google Scholar
25. Jansen R, Briaire J, Smith HE, Dom P, Haesebrouckk F, et al. (1995) Knockout mutants of *Actinobacillus pleuropneumoniae* serotype 1 that are devoid of RTX toxins do not activate or kill porcine neutrophils. Infect Immun 63: 27-37. » CrossRef » Pubmed » Google Scholar
26. Jeffery CJ (1999) Moonlighting proteins. Trends Biochem Sci 24: 8-11. » CrossRef » Pubmed » Google Scholar
27. Jenner RG, Young RA (2005) Insights into host responses against pathogens from transcriptional profiling. Nat Rev Microbiol 3: 281-294. » Pubmed » Google Scholar
28. Kadioglu A, Weiser JN, Paton JC, Andrew PW (2008) The role of *Streptococcus pneumoniae* virulence factors in host respiratory colonization and disease. Nat Rew Microbiol 6: 288-301. » Pubmed » Google Scholar
29. Karlyshev AV, Oyston PCF, Williams K, Clark GC, Titball RW, et al. (2001) Application of High-Density Array-Based Signature-Tagged Mutagenesis To Discover Novel Yersinia Virulence-Associated Genes. Infect Immun 69: 7810-7819. » CrossRef » Pubmed » Google Scholar
30. Kim JW, Dang CV (2005) Multifaceted roles of glycolytic enzymes. Trends Biochem Sci 30: 142-150. » CrossRef » Pubmed » Google Scholar
31. Lehoux DE, Sanschagrin F, Levesque RC (2002) Identification of in vivo essential genes from *Pseudomonas aeruginosa* by PCR-based signature-tagged mutagenesis. FEMS Microbiol Lett 210: 73-80. » CrossRef » Pubmed » Google Scholar

32. Mahan MJ, Slauch JM, Mekalanos JJ (1993) Selection of bacterial virulence genes that are specifically induced in host tissues. Science 259: 686-688. » Pubmed » Google Scholar

33. Mahony JB (2002) *Chlamydiae* host cell interactions revealed using DNA microarrays. Ann N Y Acad Sci 975: 192-201. » CrossRef » Pubmed » Google Scholar

34. Maione D, Margarit I, Rinaudo CD, Masignani V, Mora M, et al. (2005) Identification of a universal Group B streptococcus vaccine by multiple genome screen. Science 309: 148-150.» CrossRef » Pubmed » Google Scholar

35. Marra A, Asundi J, Bartilson M, Lawson S, Fang F, et al. (2002) Differential Fluorescence Induction Analysis of *Streptococcus pneumoniae*. Identifies Genes Involved in Pathogenesis. Infect Immun 70: 1422-1433. » CrossRef » Pubmed » Google Scholar

36. Marra A (2003) Pathogenesis genes as novel targets. In Dougherty et al. (eds) Microbial Genomics and Drug Discovery. Marcel Dekker Inc NY pp97-110.

37. Moser RJ, Reverter A, Kerr CA, Beh KJ, Lehnert SA (2004) A mixed-model approach for the analysis of cDNA microarray gene expression data from extreme-performing pigs after infection with *Actinobacillus pleuropneumoniae*. J Anim Sci 82: 1261-1271. » CrossRef » Pubmed » Google Scholar

38. Orihuela CJ, Radin JN, Sublett JE, Gao G, Kaushal D, et al. (2004) Microarray analysis of pneumococcal gene expression during invasive disease. Infect Immun 72: 5582-5596. » CrossRef » Pubmed » Google Scholar

39. Paine K, Flower DR (2002) Bacterial Bioinformatics: Pathogenesis and the Genome. J Mol Microbol Biotechnol 4: 357-365. » Pubmed » Google Scholar

40. Parkhill J, Wren BW, Thomson NR, Titball RW, Holden MT, et al. (2001) Genome sequence of *Yersinia pestis*, the causative agent of plague. Nature 413: 523-527. » CrossRef » Pubmed » Google Scholar

41. Paustian ML, May BJ, Cao D, Boley D, Kapur V (2002) Transcriptional Response of *Pasteurella multocida* to Defined Iron Sources. J Bacteriol 184: 6714-6720. » CrossRef » Pubmed » Google Scholar

42. Prentice AM (2008) Iron metabolism, malaria, and other infections: what is all the fuss about? J Nutr 138: 2537-41.» CrossRef » Pubmed » Google Scholar

43. Pich QO, Burgos R, Ferrer NM, Querol E, Piñol J (2006) *Mycoplasma genitalium* mg200 and mg386 are related with gliding motiliity and not with cytoadherence. Mol Microbiol 60: 1509-1519.

44. Piñol J, Bru S, Espuña E, Querol E (2002) Live attenuated vaccine against porcine pleuropneumoniae. EP1578210A1.

45. Pizza M, Scarlato V, Masignani V, Giuliani MM, Arico B, et al. (2000) Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science 287: 1816-1820. » CrossRef » Pubmed » Google Scholar

46. Puigbo P, Guzmán E, Romeu A, García Vallvé S (2007) OPTIMIZER: a web server for optimizing the codon usage of DNA sequences. Nucleic Acids Res 35: W126-W131.» CrossRef » Pubmed » Google Scholar

47. Reimer D, Frey J, Jansen R, Veit HP, Inzana TJ (1995) Molecular investigation of the role of ApxI and ApxII in the virulence of *Actinobacillus pleuropneumoniae* serotype 5. Microb Pathog 18: 197-209. » Pubmed

48. Rodríguez-Ortega MJ, Norais N, Bensi G, Liberatori S, Capo S, et al. (2006) Characterization and identification of vaccine candidate proteins through analysis of the group A Streptococcus surface proteome. Nat Biotechnol 24: 191-197. » CrossRef » Pubmed » Google Scholar

49. Scarselli M, Giuliani MM, Adu BJ, Pizza M, Rappuoli R (2005) The impact of genomics on vaccine design. Trends Biotechnol 23: 84-91.» CrossRef » Pubmed » Google Scholar

50. Sheehan BJ, Bosse JT, Beddek AJ, Rycroft AN, Kroll JS, et al. (2003) Identification of *Actinobacillus pleuropneumoniae* Genes Important for survival during Infection in Its natural host. Infect Immun 71: 3960-3970. » CrossRef » Pubmed » Google Scholar

51. Strauss EJ, Falkow S (1997) Microbial Pathogenesis: Genomics and Beyond. Science 276: 707-712. » CrossRef » Pubmed » Google Scholar

52. Valdivia RH, Falkow S (1997) Fluorescence-based isolation of bacterial genes expressed within host cells. Science 277: 2007-2011.» CrossRef » Pubmed » Google Scholar

53. Wagner TK, Mulks MH (2007) Identification of the *Actinobacillus pleuropneumoniae* Leucine-Responsive Regulatory Protein and Its Involvement in the Regulation of In Vivo-Induced Genes. Infect Immun. 75: 91-103. » CrossRef » Pubmed » Google Scholar

54. Wang J, Mushegiant A, Loryt S, Jin S (1996) Large-scale isolation of candidate virulence genes of

Pseudomonas aeruginosa by in vivo selection. Proc Natl Acad Sci USA 93: 10434-10439. » CrossRef » Pubmed » Google Scholar

55. Wassenaar TM, Gaastra W (2001) Bacterial virulence: can we draw the line? FEMS Microbiol Lett. 201: 1-7. » CrossRef » Pubmed » Google Scholar

56. Woods DE (2004) Comparative genomic analysis of *Pseudomonas aeruginosa* virulence. Trends Microbiol 12**:** 437-439. » CrossRef » Pubmed

57. Zhou CEZ, Lam MW, Smith JR, Zemla AT, Dyer MD, et al. (2006) MannDB - a microbial database of automated protein sequence analyses and evidence integration for protein characterization. BMC Bioinformatics 7: 459. » CrossRef » Pubmed

58. Zhou CE, Smith J, Lam M, Zemla A, Dyer MD, et al. (2007) MvirDB a microbial database of protein toxins, virulence factors and antibiotic resistance genes for bio-defence applications. Nucleic Acids Res 35: D391-94. » CrossRef » Pubmed » Google Scholar