microbial biotechnology

Microbial Biotechnology (2012) **5**(5), 594–598 doi:10.1111/j.1751-7915.2012.00353.x

Patho-Genes.org: a website dedicated to gene sequences of potential bioterror bacteria and PCR primers used to amplify them

Julien Gardès,* Dipankar Bachar, Olivier Croce and Richard Christen
CNRS UMR 7138 Systématique Adaptation et Evolution, Université de Nice-Sophia Antipolis, Parc Valrose BP71, F06108 Nice cedex 02, France.

Summary

Pathogenic agents can be very hard to detect, and usually they do not cause illness for several hours or days. To improve the speed and the accuracy of detection tests and satisfy the needs of early diagnosis, molecular biology methods such as PCR are now used. However, selecting a proper target gene and designing good primers is often not easy. We present a dedicated website, http://patho-genes.org, where we provide every sequence, functional annotation, published primer and relevant article for every annotated gene of major pathogenic bacterial species listed as key agents to be used for a bioterrorism attack. Each published primer was analysed to determine its melting temperature, its specificity and its coverage (i.e. its sensitivity against every allele of its target gene). Data generated have been organized in the form of data sheet for each gene, which are available through multiple browser panels and query systems.

Introduction

The detection protocols of pathogens are traditionally based on cell cultures and biochemical tests (or use of antibodies), which are inexpensive and largely automated. However, these procedures often require a long time. The advent of the polymerase chain reaction (PCR) allowed the emergence of molecular diagnoses. Faster [7 min (Belgrader *et al.*, 1999) or less (http://www.nhdiag.com/profile_one.shtml)] and often more accurate (allowing to characterize which alleles are present), molecular detections are progressively replacing cell cultures and biochemical tests (Christen, 2008; Fricke *et al.*, 2009), allowing early diagnosis (Saravolatz *et al.*, 2003) and therapy monitoring (Zaph, 2010).

Nevertheless, the development of molecular techniques requires selecting the proper target gene and using good primers. A valid primer should be specific of the target species and should cover every intraspecific genetic variation of the target gene. Its melting temperature (Tm) should be 55°C or above for an optimized amplification (Arun and Saurabha, 2003). *In silico* analyses theoretically allow verifying the specificity, the sensitivity and the thermodynamic conditions of a molecular protocol, before doing the real experiment.

Retrieving every sequence for a given gene is not always easy. Two different strategies are commonly used, retrieval by sequence similarity or by gene names (keywords). The most popular program to search sequences by similarity is BLAST (Altschul *et al.*, 1990). However, selecting the threshold (expect value, *e*-value) to avoid noise (similar but different genes) may be quite difficult. This *e*-value depends upon the size of the database and the proper value to select is not known *a priori*. When retrieving sequences using keywords, the difficulty lies in non-annotated gene sequences, variations in naming, or wrong annotations. Finally, retrieval of PCR primers from the literature is extremely tedious because hundreds of articles have to be carefully read. As a result, primers used are often retrieved from a recent publication but with no real warranty of specificity and coverage.

We propose a web resource, http://patho-genes.org, which includes every genetic information required for developing or using molecular detection: every sequence, functional annotation, relevant article, published primer for each pathogenicity gene of major potential bioterror bacteria. For each published primer, an *in silico* analysis was performed to determine the specificity, coverage and melting temperature of each primer. Navigation panels and query systems allow retrieving these datasets.

Results and discussion

Patho-Genes.org: Content

Data provided by this website are freely available. One can choose a pathogenic bacterium from the homepage by selecting a species or by providing primers. Table 1 lists the main information available. A browsing system allows consulting the data sheet of a given gene (Fig. 1). Genes can be selected from an alphabetical list of gene or

Received 28 September, 2011; revised 24 April, 2012; accepted 15 May, 2012. *For correspondence. Email jgardes@gmail.com; Tel. (+33) 4 92 07 69 47; Fax (+33) 4 92 07 64 05.

Table 1. Major potential bioterror bacteria and statistics at Patho-Genes.org**.**

	Public Databases			**Patho-Genes.org**			
Organism	CDS	CDS/ genome	GC%	Entries	CDS	Published primers	Articles
Bacillus anthracis	28 835	5288	34.8	1190	6 481	204	3 934
Burkholderia mallei	21 292	5213*	68.6*	1426	6 413	0	58
Burkholderia pseudomallei	31 752	6152*	68.2*	2171	11 403	0	725
Chlamydophila psittaci	2 248	963	38.9	412	1 124	103	224
Coxiella burnetti	11 590	1903	42.6	846	5 047	0	38
Francisella tularensis	14 470	1698	32.2	886	8 135	117	1 965
Mycobacterium tuberculosis	22 828	4046	65.6	1814	11 886	0	13 632
Rickettsia prowazekii	2 067	893	29.0	836	1 932	0	178
Yersinia pestis	43 032	3863	46.9	1056	31 700	187	3 307

Entries of Patho-Genes.org correspond to annotated genes for each species. CDS: number of CDS sequences available in the EMBL database. CDS/genome: average of CDS per genome. Entries: number of data sheets of genes at Patho-Genes.org. CDS: number of CDS sequences in Patho-Genes.org. Published primers: number of published primers collected from the literature. Articles: number of relevant articles found. *B. mallei* and *B. pseudomallei* genomes are composed of two chromosomes. Asterisks indicate that the average of CDS/genome for these species is the sum of averages of CDS/chromosome (GC% is an average of over the two chromosomes).

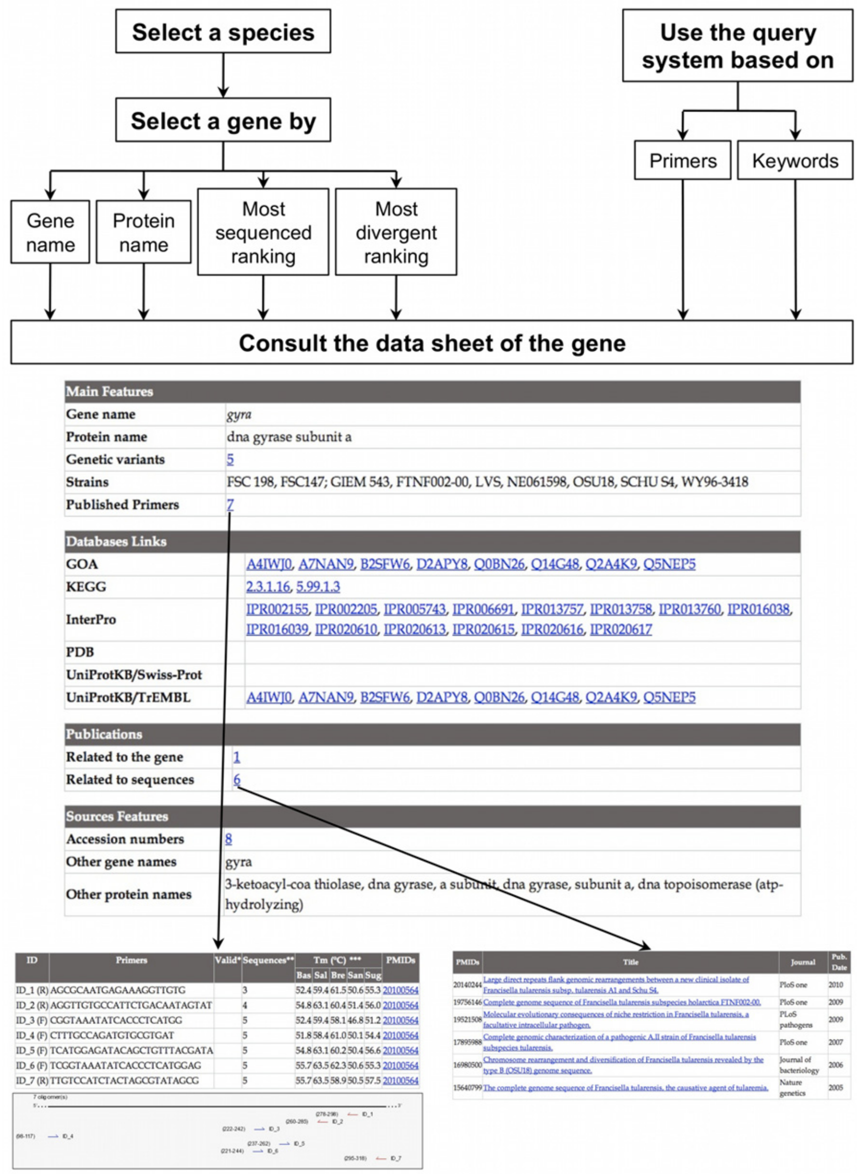
protein names, or from a ranked list of the most sequenced or the most divergent genes. The ranking of most sequenced genes is based on the number of sequences available in INSDC databases (the International Nucleotide Sequence Databases Collaboration between Japanese, European and American nucleotide databases, respectively, DDBJ, ENA and GenBank). The ranking of most divergent genes is based on the number of unique sequences for a gene, giving a rough idea of its intraspecific divergence.

Patho-Genes.org: Utility

Patho-Genes aims at providing a user-friendly web resource to ease the studies of genes and the design of PCR protocols for detecting major pathogenic bacteria (Table 1). We restricted our analysis to protein CoDing Sequences (CDS). Non-coding parts are less conserved than a CDS, and are likely to be less efficient in coverage. We standardized the display as a single gene name and a single protein name. This nomenclature was conserved between species so that the same gene name and the same protein name is used whatever the selected species (e.g. *gyrA* for the DNA gyrase subunit A), like in the HUGO Gene Nomenclature Committee with human genes (Seal *et al.*, 2011). A table containing all unified gene and protein names and their list of synonyms is available (and queries can be done using any of these alternate names). For each gene we also provide every published PCR primer extracted from the scientific literature. Users have at their disposal every data (sequences, unique sequences and alignments in FASTA format) to design new PCR primers or to test the validity of known PCR primers, for example via Primer3 (Untergasser *et al.*, 2007) or Prifi (Fredslund *et al.*, 2005) or the web application OHM (Croce *et al.*, 2008).

The most sequenced or the most divergent ranked results are also useful to start a *de novo* primer design or to re-orient a strategy of detection. This can ease the selection of a target gene, for example by selecting most studied and conserved genes for a higher coverage. However, genes having several copies in the same genome can bias these rankings. Indeed, our method of collecting sequences by similarity gathers, in a single cluster, identical or nearly identical genes whose loci are different. It is typically the case of housekeeping genes from transposons and other selfish genetic elements (e.g. transposases), which are known to be present in several copies in bacterial genomes (Gibert *et al*., 1990; Beuzón *et al.*, 2004). Although ranks may be overvalued, this type of clustering was retained because these different loci correspond to copies of the same gene and the difference between sequences is limited to a few single nucleotide polymorphisms (SNPs).

From the data sheet of a gene (Fig. 1), users have access to several resources such as strains sequenced for the gene, links to the main biological databases (KEGG, GO, Interpro, PDB and Uniprot), links to the NCBI sequence viewer and relevant publications. It is also possible to download FASTA files containing only unique sequences, aligned, with published primers or every sequence for a gene. A text file, compatible with regular spreadsheet, is available with the coordinates and the strain name of each sequence. Each published PCR primer is displayed with Tms computed using five different methods, its position within aligned sequences, estimates of coverage and specificity, as well as links to publications in which they were found. Some genes had a majority of forward/reverse primers or only one published primer, although a minimum of two is required for PCR amplification. These results were seemingly caused by a design in non-coding regions, by the pres-



Main Features	
Gene name	*gyra*
Protein name	dna gyrase subunit a
Genetic variants	5
Strains	FSC 198, FSC147; GIEM 543, FTNF002-00, LVS, NE061598, OSU18, SCHU S4, WY96-3418
Published Primers	7

Databases Links	
GOA	A4IWJ0, A7NAN9, B2SFW6, D2APY8, Q0BN26, Q14G48, Q2A4K9, Q5NEP5
KEGG	2.3.1.16, 5.99.1.3
InterPro	IPR002155, IPR002205, IPR005743, IPR006691, IPR013757, IPR013758, IPR013760, IPR016038, IPR016039, IPR020610, IPR020613, IPR020615, IPR020616, IPR020617
PDB	
UniProtKB/Swiss-Prot	
UniProtKB/TrEMBL	A4IWJ0, A7NAN9, B2SFW6, D2APY8, Q0BN26, Q14G48, Q2A4K9, Q5NEP5

Publications	
Related to the gene	1
Related to sequences	6

Sources Features	
Accession numbers	8
Other gene names	gyra
Other protein names	3-ketoacyl-coa thiolase, dna gyrase, a subunit, dna gyrase, subunit a, dna topoisomerase (atp-hydrolyzing)

ID	Primers	Valid*	Sequences**	Tm (°C) ***					PMIDs
				Bas	Sal	Bre	San	Sug	
ID_1 (R)	AGCGCAATGAGAAAGGTTGTG		3	52.4	59.4	61.5	50.6	55.3	20100564
ID_2 (R)	AGGTTGTGCCATTCTGACAATAGTAT		4	54.8	63.1	60.4	51.4	56.0	20100564
ID_3 (F)	CGGTAAATATCACCCTCATGG		5	52.4	59.4	58.1	46.8	51.2	20100564
ID_4 (F)	CTTTGCCAGATGTGCGTGAT		5	51.8	58.4	61.0	50.1	54.4	20100564
ID_5 (F)	TCATGGAGATACAGCTGTTTACGATA		5	54.8	63.1	60.2	50.4	56.6	20100564
ID_6 (F)	TCGGTAAATATCACCCTCATGGAG		5	55.7	63.5	62.3	50.6	55.3	20100564
ID_7 (R)	TTGTCCATCTACTAGCGTATAGCG		5	55.7	63.5	58.9	50.5	57.5	20100564

PMIDs	Title	Journal	Pub. Date
20140244	Large direct repeats flank genomic rearrangements between a new clinical isolate of Francisella tularensis subsp. tularensis A1 and Schu S4.	PloS one	2010
19756146	Complete genome sequence of Francisella tularensis subspecies holarctica FTNF002-00.	PloS one	2009
19521508	Molecular evolutionary consequences of niche restriction in Francisella tularensis, a facultative intracellular pathogen.	PLoS pathogens	2009
17895988	Complete genomic characterization of a pathogenic A.II strain of Francisella tularensis subspecies tularensis.	PloS one	2007
16980500	Chromosome rearrangement and diversification of Francisella tularensis revealed by the type B (OSU18) genome sequence.	Journal of bacteriology	2006
15640799	The complete genome sequence of Francisella tularensis, the causative agent of tularemia.	Nature genetics	2005

Fig. 1. Example of resources available in Patho-Genes. After selecting a species, users can find a gene via browsing panel located at the top of the species homepage. Genes can be selected by gene name, protein name, or by rank of most sequenced or most divergent genes. Two query systems allow using either keywords or primers to access the data sheet. From the data sheet, users can download sequences at FASTA format [every sequence (accession numbers), unique sequence (genetic variants) or aligned sequences], published primers (Tm and localization) or relevant articles.

ence of an additional restriction site added to the primers leading to the failure of our automated retrieval process, or finally when a larger genomic fragment was amplified with primers located within two different genes (Gardès *et al*., 2012). However, users can combine two primers thanks to the alignment map of primers with their target gene, when several primers are available.

Patho-Genes.org: Tools

From the navigation panel, two query systems are available in Patho-Genes. First, entering a gene, a protein or an author name or selecting a species or a group of species allows searching for genes. Using the option 'include alternate names', it is possible to find every sequence of a gene using any alternate name used to describe a gene. To select only genes having published primers, users can check the case 'published primer'. With this action and by not filling the sections gene, protein and author names, the list of genes having published primers for a species or for the whole database can be displayed too. Unlike current online retrieval system by keywords, our query system retrieves even non-annotated sequences of a gene because of the combination of similarity and keywords used to build this database. Second, querying by using a primer or a couple of primer's sequences allows retrieving target genes, the amplicon sequences and the positions of primers in aligned sequences.

Patho-Genes.org: Conclusions

Patho-Genes.org is a website containing every information useful for the development of molecular detection tests based on PCR and the study of genes in major potential bioterror bacteria. Contrary to Multilocus Sequence Typing (MLST) databases (e.g. http://www.mlst.net/ or http://pubmlst.org/) that focus on seven housekeeping genes per species by proposing every allele, couples of primers and MLST profiles, Patho-Genes.org targets every annotated gene of a species but does not provide any MLST profile. In particular, our platform offers an analysis of the quality of published primers. Indeed, in the case of *Vibrio cholerae*, we discovered that only one-third of published PCR primers are able to amplify every allele of their target gene (Gardès *et al.*, 2012). This tool thus allows to rapidly check the main characteristics of primers (specificity, coverage and thermodynamic parameters). Presently, primers are available only for four species. Primers for the other species of Table 1 will be available soon.

Methods

Ethics Statement: this study did not involve any living being or biological sample

Protein coding DNA sequences belonging to each potential pathogenic bacterium were collected using the ACNUC database and its retrieval system (Gouy and Delmotte, 2008). For each species, the occurrence of gene names was computed to obtain a raw list of most frequent names. From this list, tBLASTx analyses were performed to cluster similar sequences. Every annotation of such similar sequences was collected and the gene was internally described using the most used gene and protein names. Relevance and consistency of each term were checked among species. For each gene, sequences were then '*de-replicated*': sequences contained into a longer sequence or identical sequences were removed in order to obtain a set of unique sequences. This set was aligned with MUSCLE version 3.8.31 (Edgar, 2004).

Using species name and annotations of similar sequences, requests were done with Entrez at NCBI (PubMed), Jane (Schuemie and Kors, 2008) and eTBLAST (Errami *et al.*, 2007) in order to retrieve a combined list of relevant PubMed IDentification numbers (PMID) for each gene. Some requests yielded up to hundreds of publications. Each article was downloaded in PDF format and relevant short nucleic acid sequences were extracted from each file using regular expressions. Oligomers found at least once in the set of unique sequences were considered as published primers. Because different methods to calculate a Tm can give different results, the basic (Mann *et al.*, 2009) (bas), the salt adjusted (Howley *et al.*, 1979) (Sal) and three nearest-neighbour (Breslauer *et al.*, 1986; Sugimoto *et al.*, 1996; SantaLucia, 1998) (Bre, San and Sug) methods were used to compute Tm of published primers via dnaMATE (Panjkovich *et al.*, 2005).

An Apache2 web server hosts Patho-Genes. From the navigation panel, two query systems are available. The first one is written in python and allows searching for genes using keywords: gene, protein or author names. It is possible to specify if the search should take in account every annotation used in INSDC databases, or be limited to genes having published primers. The program checks the existence of submitted keywords and selects corresponding entries. The second query system is based on a C program developed by our team, which returns genes containing a primer or a set of primers. Unlike BLAST, this software allows the use of the IUPAC code for degenerate positions.

Future directions

Patho-Genes will be updated regularly to incorporate the last release of the public databases. In the future, other organisms, like pulmonary pathogens or new emerging pathogens, will be included. The main goal of this project is to provide an exhaustive and user-friendly web resources for DNA-based detection technologies of pathogenic bacteria.

Acknowledgements

Our study was supported by a PhD fellowship from the Direction Générale de l'Armement (DGA), Ministère de la Défense Française. We thank the two referees for constructive comments that allowed us to improve this manuscript and our website.

References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Arun, A., and Saurabha, D. (2003) PCR primer design. In *PCR Primer: A Laboratory Manual*. Dieffenbach, C.W., and Dveksler, G.S. (eds). New York, USA: Cold Spring Harbor Laboratory Press, pp. 61–64.

Belgrader, P., Benett, W., Hadley, D., Richards, J., Stratton, P., Mariella, R., and Milanovich, F. (1999) PCR detection of bacteria in seven minutes. *Science* **284:** 449–450.

Beuzón, C.R., Chessa, D., and Casadesús, J. (2004) IS200: an old and still bacterial transposon. *Int Microbiol* **7:** 3–12.

Breslauer, K.J., Frank, R., Blöcker, H., and Marky, L.A. (1986) Predicting DNA duplex stability from the base sequence. *Proc Natl Acad Sci USA* **83:** 3746–3750.

Christen, R. (2008) Identifications of pathogens – a bioinformatic point of view. *Curr Opin Biotechnol* **19:** 266–273.

Croce, O., Chevenet, F., and Christen, R. (2008) OligoHeatMap (OHM): an online tool to estimate and display hybridizations of oligonucleotides onto DNA sequences. *Nucleic Acids Res* **36:** W154–W156.

Edgar, R.C. (2004) MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinformatics* **5:** 113.

Errami, M., Wren, J.D., Hicks, J.M., and Garner, H.R. (2007) eTBLAST: a web server to identify expert reviewers, appropriate journals and similar publications. *Nucleic Acids Res* **35:** W12–W15.

Fredslund, J., Schauser, L., Madsen, L.H., Sandal, N., and Stougaard, J. (2005) PriFi: using a multiple alignment of related sequences to find primers for amplification of homologs. *Nucleic Acids Res* **33:** W516–W520.

Fricke, W.F., Rasko, D.A., and Ravel, J. (2009) The role of genomics in the identification, prediction, and prevention of biological threats. *PLoS Biol* **7:** e1000217.

Gardès, J., Croce, O., and Christen, R. (2012) *In silico* analyses of primers used to detect the pathogenicity genes of *Vibrio cholerae* [WWW document]. URL https://www.jstage.jst.go.jp/article/jsme2/advpub/0/advpub_ME11317/_article.

Gibert, I., Barbé, J., and Casadesús, J. (1990) Distribution of insertion sequence IS200 in *Salmonella* and *Shigella*. *J Gen Microbiol* **136:** 2555–2560.

Gouy, M., and Delmotte, S. (2008) Remote access to ACNUC nucleotide and protein sequence databases at PBIL. *Biochimie* **90:** 555–562.

Howley, P.M., Israel, M.A., Law, M.F., and Martin, M.A. (1979) A rapid method for detecting and mapping homology between heterologous DNAs. Evaluation of polyomavirus genomes. *J Biol Chem* **254:** 4876–4883.

Mann, T., Humbert, R., Dorschner, M., Stamatoyannopoulos, J., and Noble, W.S. (2009) A thermodynamic approach to PCR primer design. *Nucleic Acids Res* **37:** e95.

Panjkovich, A., Norambuena, T., and Melo, F. (2005) dnaMATE: a consensus melting temperature prediction server for short DNA sequences. *Nucleic Acids Res* **33:** W570–W572.

SantaLucia, J. (1998) A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc Natl Acad Sci USA* **95:** 1460–1465.

Saravolatz, L.D., Manzor, O., VanderVelde, N., Pawlak, J., and Belian, B. (2003) Broad-range bacterial polymerase chain reaction for early detection of bacterial meningitis. *Clin Infect Dis* **36:** 40–45.

Schuemie, M.J., and Kors, J.A. (2008) Jane: suggesting journals, finding experts. *Bioinformatics* **24:** 727–728.

Seal, R.L., Gordon, S.M., Lush, M.J., Wright, M.W., and Bruford, E.A. (2011) genenames.org: the HGNC resources in 2011. *Nucleic Acids Res* **39:** D514–D519.

Sugimoto, N., Nakano, S., Yoneyama, M., and Honda, K. (1996) Improved thermodynamic parameters and helix initiation factor to predict stability of DNA duplexes. *Nucleic Acids Res* **24:** 4501–4505.

Untergasser, A., Nijveen, H., Rao, X., Bisseling, T., Geurts, R., and Leunissen, J.A.M. (2007) Primer3Plus, an enhanced web interface to Primer3. *Nucleic Acids Res* **35:** W71–W74.

Zaph, C. (2010) Which species are in your feces? *J Clin Invest* **120:** 4182–4185.