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In silico analysis reveals the role of horizontally transferred genes (HGTs) in shaping the pathogenicity of *Xanthomonas*

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The role of *Xanthomonas* spp. as phytopathogen has been well recognized in diseases of important crops like rice, canola, tomato, citrus, etc. The genomes of a number of *Xanthomonas* strains are also fully sequenced and they are made available in various data bases. In the present study, *in silico* analysis of six *Xanthomonas* genomes was carried out. Synonymous codon usage pattern study in these genomes revealed that pathogenicity related (PR)-horizontally transferred genes (HGTs) were, in general, expressed lowly and were less biased in comparison with average protein coding genes and ribosomal protein genes. Moreover, the correspondence analysis showed that the ribosomal genes were clustered at one end, while the HGTs and the PR-HGTs were all scattered. It has been also revealed that how the virulent HGTs, in spite of having low expression levels, did enhance the pathogenicity of the *Xanthomonas* strains to certain extent by targeting important cellular functions.

Keywords: Codon adaptation index, codon usage, correspondence analysis, horizontally transferred genes, pathogenicity, *Xanthomonas*

Introduction

The genus *Xanthomonas*, a member of the Proteobacteria[1], is a Gram-negative, non-fermentative, rod-shaped, flagellated, obligately aerobic organism and is known to produce brominated yellow coloured pigment called xanthomonadin[2]. Almost all the reported strains of this genus are plant-associated and are known to cause diseases in agriculturally important plant crops. Among the prominent *Xanthomonas* species are *X. oryzae* (a pathogen of rice), *X. axonopodis* (causes bacterial canker in citrus species) and *X. campestris* (responsible for bacterial black spot in various crops like capsicum, tomato and canola). Genome sequences of all major *Xanthomonas* species are now available, which gives the researchers the opportunity to study various aspects of these genomes *in silico*.

The lateral gene transfer, most commonly known as the horizontal gene transfer (HGT), is a process whereby the genetic material of one organism gets incorporated into another organism, irrespective of the fact that the latter is not the offspring of the former. This phenomenon is very common in bacteria, even between distantly related strains. It was in the year 1959 when the process of HGT was first described in Japan[3]. In case of prokaryotes, the horizontal transfer of genes is brought about by mainly three mechanisms, *viz*., transformation, conjugation and transduction. This phenomenon of transfer not only helps in the acquisition of new genes that give rise to novel phenotypic characteristics, but also plays important role from evolutionary point of view. In pathogenic species, it helps in the maintenance and enhancement of virulence and also in the spread of drug resistance[3].

Novel virulence factors can be identified with the help of genome analyses and comparative genomics[4], which has brought about a paradigm shift in the research of bacterial pathogenesis[5]. Within the genus of *Xanthomonas*, several genes have been found to be associated with pathogenicity and virulence and are commonly known as pathogenicity related (PR) genes[6]. Among the PR genes, the *hrp* (hypersensitive reaction and pathogenicity) genes govern the interactions between plant pathogens and their host

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plants[7]. The *gum* genes (encode for a protein called xanthan gum), the interruption of which leads to reduced virulence in *Xanthomonas* sp.[9]. The *avr* (avirulence) genes, on the other hand, encode for a group of elicitors. These elicitors or effectors help bacteria to initiate hypersensitive response in the resistant hosts, thereby establishing pathogenicity in these plants[10].

Comparative genomics have shown that DNA islands containing the PR genes have largely been acquired by means of HGT by a number of pathogens like *Escherichia coli*, *Salmonella*, *Helicobacter pylori*, etc.[11]. In the present study, authors have determined the expression level of protein coding genes in the phytopathogenic species of *Xanthomonas.* They have also analyzed those PR genes that are transferred horizontally and their possible role in enhancing virulence.

Materials and Methods

Six strains of *Xanthomonas*, *viz*., *Xanthomonas axonopodis* pv. *citri* 306 (XAC), *X. oryzae* pv. *oryzae* MAFF 311018 (XOO2), *X. oryzae* pv. *oryzae* KACC 10331 (XOO1), *X. campestris* pv. *campestris* 8004 (XCC1), *X. campestris* pv. *campestris* ATCC 33913 (XCC2) and *X. campestris* pv. *vesicatoria* 85-10 (XCV), were used and their complete genome sequences were obtained from the IMG database (http://img.jgi.doe.gov). Among these strains, XAC and XCV possess plasmids[12].

In order to analyze the codon usage bias, a number of parameters have been used. The Nc (effective number of codon) was used to compute the general codon bias[12,13]. The Nc value is actually a depiction of the quantity of equal codons that would generate the same codon usage bias. The GC3s denotes the occurrence of G and C at the synonymous third position. Another index, CBI (codon bias index)[14] measures the extent to which a gene uses a proportion of optimal codon. The Fop (frequency of optimal codons)[15,16] is the fraction of synonymous codons that are optimal codons. The RSCU (relative synonymous codon usage) is the proportion of the observed frequency of a codon to the expected frequency in case, when all the synonymous codons for the amino acids are used in equal share. All the above parameters were calculated using the codonW software (bioweb2.pasteur.fr/seqanal/interfaces/codonw). CAI (codon adaptation index) is a parameter for measurement of deviation of a given protein coding gene sequence with respect to a reference set[17]. CAI was determined using ACUA[18] keeping ribosomal protein genes as reference. The higher CAI values indicate that the codon usage pattern of the genes of interest is more similar to the reference genes.

To know whether the PR-HGTs are present in all the strains or native to a particular strain, an analysis of these genes was carried out. Information regarding the HGT was obtained from the website: http://cbcsrv.watson.ibm.com/HGT_SVM/. The PR-HGTs were sorted out as per Goyal *et al*[19]. Briefly, the sorted genes were subjected to IMG Genomes database to sort out the sequence homologs with respect to all the studied strains setting minimum per cent identity and maximum E (except) values at 90% and le-2, respectively.

Results and Discussion

Analysis of Codon Usage Patterns of PR-HGTs

The six isolates of *Xanthomonas* included in the present study, *i.e*., XAC, XCC1, XCC2, XCV, XOO1 and XOO2, have been reported to possess 558, 555, 543, 564, 533 and 617 HGTs and 67, 71, 49, 61, 58 and 55 PR-HGTs, respectively (http://cbcsrv.watson.ibm.com/HGT_SVM/). Comparing these two, it is clear that there are number of genes those are both pathogenicity related as well as horizontally transferred (Table 1).

Generally, amongst genes in the same genome, each organism has its own codon usage pattern, which varies greatly with the other genome. These variations can be best determined by means of a plot between effective numbers of codons (Nc) *versus* GC3. Earlier, it had been noticed that, in case of genes that were highly expressed, translational selection played a major role on the codon bias; whereas in case of lowly expressed genes, it was the mutational bias that played a major role[20]. In the present study, an Nc/GC3 plot for protein coding genes of all the six strains of *Xanthomonas* is shown in Fig. 1. It is clear from the plot that a substantial variation of codon usage exists in *Xanthomonas*, which is evident from the Nc values ranging from 22±2 to 61±0 for all genomes. The ribosomal genes, whose expression levels are assumed to be high during the growth period, have also been decorated in the plot. These genes remain clustered towards the lower end of the plot, thus suggesting that a strong codon bias acts upon by the translational selection[21]. The HTGs have been highlighted in the plot and they fall below the

Table 1—Pathogenicity related gene products analyzed in *Xanthomonas* strains

Strains	Category of PR-HGTs	Gene products
XAC	*vir* gene products	VirB1,VirB2,VirB3,VirB4, VirB6,VirB8,VirB9,VirB10, VirB11,VirD4
	gum gene products	GumC, GumE, GumK, GumL
	hrp associated proteins	HrpD5, HrpE ,HrpF, HrpW
	Avirulence protein	AvrXacE1, AvrXacE2
	xrvA gene product	H-NS family nucleoid protein XrvA
XOO2	hrp associated proteins	HrpE1, HrpF, HrpG
	gum gene products	GumB precursor, GumC, GumE, GumF, GumG, GumH, glucoronosyltransferase GumK, GumL
	xrvA gene product	H-NS family nucleoid protein XrvA
XOO1	hrp associated protein	HrpC3, HrpD2, HrpD5, HrpE, HrpF, HrpG
	gum gene products	GumB, GumC, GumE, GumF, GumH, GumL, glucoronosyltransferase GumK
	Avirulence protein	AvrXa3
	xrvA gene product	H-NS family nucleoid protein XrvA
	vir gene products	VirK
XCC1	*vir* gene products	VirB1,VirB2,VirB3,VirB4, VirD4,VirB6,VirB8,VirB9, VirB10,VirB11,VirK,VirP
	gum gene products	GumB, GumC, GumD, GumE, GumF, GumG, GumH, GumI, GumJ, GumK, GumL, GumM, GumN
		Virulence and avirulence proteins
	xrvA gene product	H-NS family nucleoid protein XrvA
XCC2	hrp associated protein	HrpB2, HrpE, HrpF, HrpG
	gum gene products	GumC, GumE, GumF, GumG, GumH, GumK, GumL
	vir gene products	VirB1,VirB2,VirB3,VirB4, VirD4,VirB6,VirB8,VirB9, VirB10,VirB11
	Avirulence gene products	AvrXccB, AvrXccA2, AvrBs1, AvrXccC, AvrBs1.1, AvrXccE1
	Virulence gene product	PsvA
XCV	hrp associated proteins	HrpE, HrpF
	gum gene products	GumK, GumL
	vir gene products	VirD4,VirB6,VirB8,VirB9
	Avirulence gene products	AvrRx01, AvrRxv

PR-HGTs= Pathogenicity related-horizontally transferred genes

expected curve. Also the genes those are pathogenic and assumed to be HTGs are plotted. From the plots, it is apparent that most of the genes lie below the GC3 curve implying that GC3 alone does not state synonymous codon bias[15]. Lower Nc values indicated elevated bias. Protein coding genes and ribosomal protein genes were more biased compared to those of the HGTs and the PR-HGTs.

In the present study, the Fop values of protein coding genes and ribosomal protein genes were found higher compared to the PR genes and HGTs (Table 2), pointing out that PR genes and HGTs had lower frequency of optimal codons. This entails the influence of mutational bias for the PR genes and HGTs. Further, ribosomal protein genes had higher mean CBI values compared to the other groups of genes; higher CBI values indicate higher bias. Analysis of the mean CAI values (Table 2) showed that ribosomal protein genes had higher CAI values than all other group of genes, except in case of XCV and XOO1 where the PR genes had higher CAI values. Higher CAI values indicate higher level of gene expression.

Multivariate Statistical Analysis

To study the variation in codon usage, multivariate statistical analysis and correspondence analysis were performed. In Fig. 2, the position of HGTs and PR-HGTs with respect to other genes in the genomes is illustrated by the first and second principal axes. It is clear from the results that the scatter plots of XCC2 and XOO2 show a small core region and two ascending horns, while the scatter plots of XAC, XCC1, XCV and XOO1 show two descending horns. From the plots, it can be well seen that the ribosomal protein genes are clustered at one end, indicating their high expression levels. The PR genes those are horizontally transferred are found scattered and not clustered, suggesting that they might have originated from different ancestors as well as in different times.

Role of Horizontally Transferred Virulent Genes in Cell Functions

Our main motto in the present study was to investigate whether HGTs play any role in shaping the pathogenicity in *Xanthomonas*. For this purpose, we divided the potentially virulent HGTs in different COG (cluster of orthologous groups of proteins) functional categories. COG denotes the comparison of protein sequences encoded in complete genome

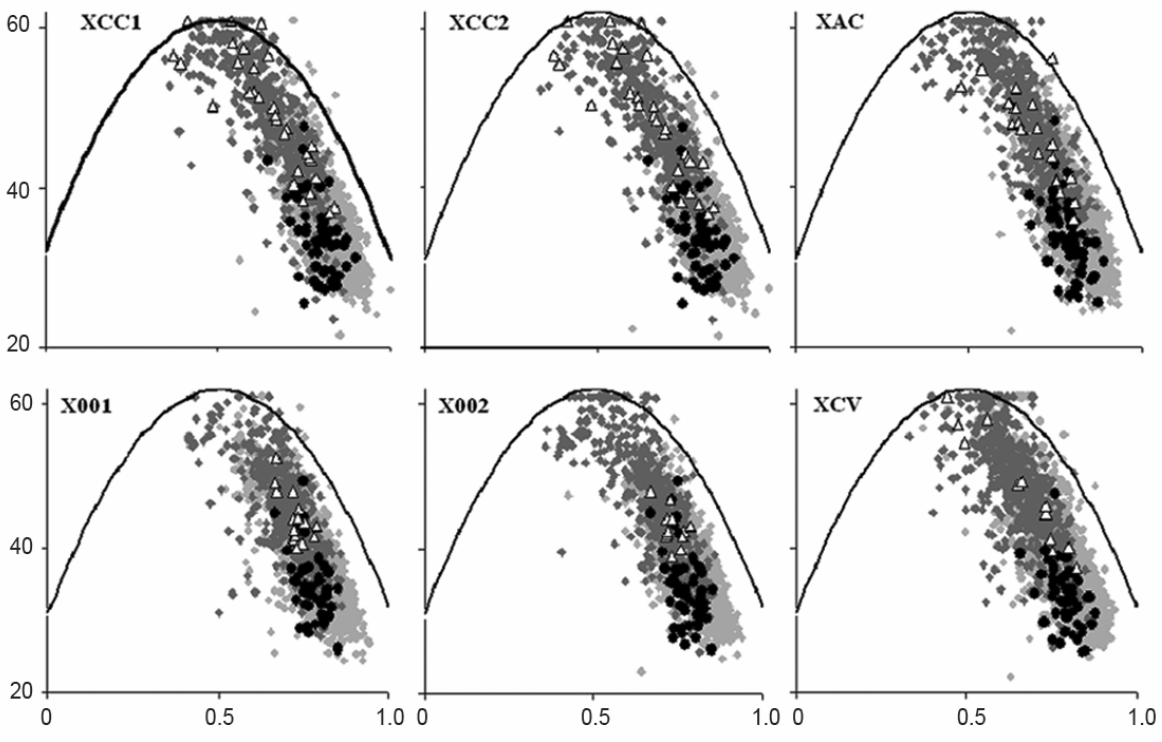

Fig. 1—Plot of the effective number of codons used (Nc) in each gene (Y axis) against the G+C content at synonymous third position of codons (GC3) (X axis) for all *Xanthomonas* genomes. The continuous curve in each plot symbolizes the null hypothesis that the GC bias at the synonymous site is solely due to mutation and not selection. (Protein coding genes=Light grey circles; Ribosomal protein genes=Black circles; HGTs=Dark grey rhombus; PR-HGTs=White triangles)

representing major phylogenetic lineages. Each COG type consists of individual protein or groups of paralogs from at least 3 lineages and corresponds to an ancient conserved domain[12]. For *Xanthomonas* genomes, we have identified 8 different COG categories and the number of potential virulent HGTs in COGs were found to be 10, 14, 15, 5, 8 and 9 in XAC, XCC1, XCC2, XCV, XOO1 and XOO2, respectively (Table 3).

The presence of virulent genes in the cell membrane (COG-M) indicates their role in pathogenesis by causing membrane perturbations. Invasion of bacteria leads to a rise in Ca^{2+}, which activates membrane bound callose synthase enzyme and finally deposition of callose in membranes, which causes membrane perturbations[22]. The *gum* genes so present are involved in the production of an exopolysaccharide in *Xanthomonas* strains called xanthan gum[6]. However, truncated xanthan acts as a suppressor in callose deposition, thereby making the plants resistant to infections[22].

The genes involved in the intracellular trafficking, secretion and vesicular transport mechanisms (COG-U) are part of the Type III and Type IV secretion systems. The *vir* gene products are Type IV secretion-associated proteins, while the HrpG (COG-K & T) is a Type III secretion-associated protein. Many pathogenic bacteria make use of these macromolecule delivery systems (Type III & Type IV) to deliver bacterial virulence proteins into the host cells[23]. Such mechanism of delivery of protein helps the pathogens to gain access to a vast number of host targets[24]. The *vir* genes involved in these mechanisms do play important roles in eliciting hypersensitive response, pathogenesis and a number of other functions in *Xanthomonas*. HrpG is a key regulator protein of the TTSS (type III secretion system) which plays important role not only in plant pathogenicity but also in eliciting hypersensitive response in plants. This HrpG is a two component response regulator protein, which controls the synthesis of *hrpX* and *hrpA* that bring about the

Table 2—Mean values of guanine cytosine ratio at third position (GC3), effective number of codons (Nc), codon adaptation index (CAI), codon bias index (CBI) and frequency of optimal codons (Fop) of the genes in the six strains of *Xanthomonas*

Organism	Genes	%GC3 (mean)	Nc (mean)	CAI (mean)	CBI (mean)	Fop (mean)
XAC	PCG	78.9	37.90	0.6674	0.2964	0.5844
	RPG	79.1	33.63	0.6963	0.3485	0.6212
	HGT	66.8	47.92	0.5092	0.1566	0.5064
	PRG	73.4	42.76	0.6588	0.2865	0.5788
	PR-HGT	68.6	46.81	0.5399	0.1814	0.5234
XOO2	PCG	77.1	39.03	0.6637	0.2810	0.5752
	RPG	77.6	34.45	0.6985	0.3431	0.6182
	HGT	67.9	46.33	0.5535	0.1870	0.5237
	PRG	77.1	39.67	0.6635	0.2694	0.5695
	PR-HGT	73.5	42.96	0.6093	0.2155	0.5383
XOO1	PCG	77.0	39.19	0.6631	0.2809	0.5748
	RPG	77.7	34.79	0.6970	0.3269	0.6091
	HGT	70.4	44.91	0.5827	0.2110	0.5368
	PRG	75.9	41.03	0.6474	0.2767	0.5721
	PR-HGT	72.5	44.18	0.5961	0.2020	0.5271
XCC1	PCG	80.2	36.76	0.6695	0.3126	0.5937
	RPG	79.2	33.88	0.6792	0.3547	0.6251
	HGT	66.2	47.78	0.6695	0.1615	0.5089
	PRG	72.5	43.21	0.6906	0.3210	0.5958
	PR-HGT	64.8	49.27	0.4869	0.1491	0.5028
XCC2	PCG	80.6	36.49	0.6712	0.3165	0.5961
	RPG	79.2	33.89	0.6765	0.3549	0.6252
	HGT	67.2	46.95	0.5007	0.1735	0.5172
	PRG	72.6	43.04	0.6808	0.3182	0.5956
	PR-HGT	65.1	48.83	0.4886	0.1479	0.5020
XCV	PCG	78.3	38.26	0.6620	0.2304	0.5813
	RPG	79.2	33.41	0.7021	0.3528	0.6244
	HGT	65.8	47.91	0.5097	0.1576	0.5076
	PRG	74.6	41.19	0.6535	0.2898	0.5783
	PR-HGT	65.7	48.16	0.5233	0.1648	0.5171

PCG=Protein coding genes; RPG=Ribosomal protein genes; HGT=Horizontal transferred genes; PRG=Pathogenicity related genes; PR-HGT=Pathogenicity related- horizontally transferred genes

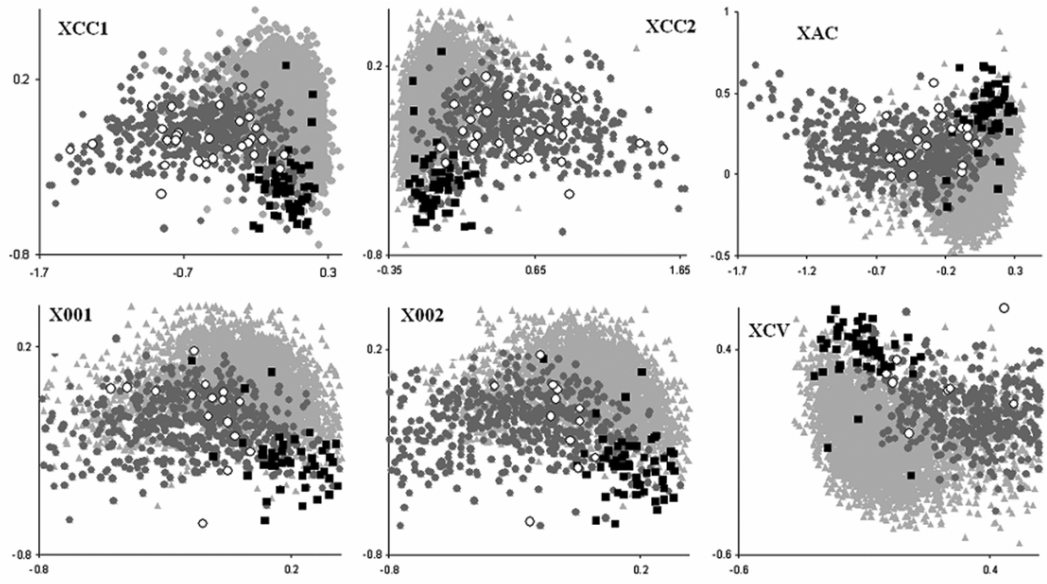

Fig. 2—Correspondence analysis of codon usage patterns of the six strains of *Xanthomonas*. In all the plots, X and Y axes correspond to axes 1 and 2 of the analysis. (Protein coding genes=Light grey; Ribosomal protein genes=Black boxes; HGTs=Dark grey circles; PR-HGTs=White circles)

Table 3—PR-HGTs of six strains of *Xanthomonas* affecting major cellular functions

Xanthomonas strains	PR-HGTs in COG* functional categories								Total#
	M	U	K	T	R	G	N	S	
XAC	*gumC*	*virB3, virB4, virD4, virB8, virB9, virB10, virB11*			*xrvA*		*virB11*		10
XCC1	*gumC, gumH, gumK*	*virB3, virB4, virD4, virB6, virB8, virB9, virB10, virB11*				*gumF, gumG*	*virB11*		14
XCC2	*gumC, gumH, gumK*	*virB3, virB4, virD4, virB8, virB9, virB10, virB11*	*hrpG*	*hrpG*		*gumF, gumG*	*virB11*		15
XCV	*gumK*	*virD4, virB6, virB8, virB9*							5
XOO1	*gumB, gumC, gumK*	*hrcR*	*hrpG*	*hrpG*	*xrvA*	*gumF*			8
XOO2	*gumB, gumC, gumH, gumK*		*hrpG*	*hrpG*	*xrvA*	*gumF*		Hrp-associated gene product	9

PR-HGTs= Pathogenicity related- horizontally transferred genes
*COG functional categories: M=Cell wall/membrane/envelope biogenesis; U=Intracellular trafficking, secretion, and vesicular transport; K=Transcription; T=Signal transduction mechanisms; R=General function prediction only; G=Carbohydrate transport and metabolism; N=Cell motility; S=Function unknown.
#Total no. of PR-HGTs in each genome

transcription of the operons *hrpB* to *hrpF*[25]. The products of these genes are involved in the pathogenesis of *Xanthomonas* strains. These genes are also found to be associated with the signal transduction mechanisms and encode a response regulator protein in the two component signal transduction system of bacteria[25]. This response regulator brings about further transcription of other *hrp* genes that are associated with pathogenesis of *Xanthomonas*.

The gene that has been found associated with general function (COG-R) is the *xrvA*. This gene encodes a histone like nucleoid structuring protein (H-NS) domain, which plays an important role in establishing virulence, generating hypersensitive response, production of exopolysaccharide and diffusible signal factor (DSF). This implies that it might encode an important regulatory factor for the virulence of *Xanthomonas*[26].

Carbohydrate transport and metabolism are important cellular functions and the *gumG* and *gumF* (COG-G) genes are associated with it. Both these genes code for an acetyltransferase enzyme. This enzyme brings about acetylation of mannose residues, which results in modification of the xanthan[27]. Synthesis begins with glucose as substrate for synthesis of sugar nucleotide precursors like UDP-glucose, UDP-glucoronate and GDP-mannose to form the pentasaccharide repeating units. This links the synthesis of xanthan to carbohydrate metabolism[28]. However, the role of the *virB*11 gene in cell motility in *Xanthomonas* has not yet been ascertained[29].

It is, therefore, apparent that the analysis of COG category genes reveales some interesting results indicating major roles of PR-HGTs in maintaining virulance in various strains of *Xanthomonas.*

Conclusion

Organisms have acquired genes by means of the horizontal transfer mechanism. In the present study, the approach was to analyse the codon usage pattern of available *Xanthomonas* genomes with special emphasis on those genes that are pathogenicity related and have been acquired by horizontal transfer. From the results, it can be said that considerable variation existed among the genes of *Xanthomonas* strains. On studying the pathogenicity related genes, it has been found that considerable number of pathogenicity related genes are virtually acquired by horizontal gene transfer mechanism. As expected horizontally transferred virulent genes are largely expressed at a lower level. However a number of such genes are involved in virulence, invasion, breaking host defense mechanism and thereby causing infections. Considerable similarities in the pathogenicity-related genes within the strains also indicate that these genes are mobile within the genus. An analysis of these horizontally transferred pathogenicity related genes have also shown how their presence in the organisms bring about important changes in the cellular functions, thus enabling the pathogens to establish themselves in the host and thereby causing disease.

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