Molecular Diversification in the Quorum-Sensing System of *Vibrio cholerae*: Role of Natural Selection in the Emergence of Pandemic Strains[▽]

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Two haplotypes of the *Vibrio cholerae* quorum-sensing system regulator *hapR* are described: *hapR1*, common among nonpandemic, non-O1, non-O139 strains, and *hapR2*, associated with pandemic O1 and O139 and epidemic O37 *V. cholerae* strains. The *hapR2* has evolved under strong natural selection, implying that its fixation was influenced by conditions that led to cholera pandemics.

There are over 200 known serotypes of *Vibrio cholerae*, but for unclear reasons, only two (serotypes O1 and O139) have caused global outbreaks of cholera (25). The majority of *V. cholerae* strains isolated from aquatic environments are non-O1 and non-O139. They are capable of causing various infections in humans, including secretory diarrhea, gastroenteritis, septicemia, and infections of the ear and skin (28, 30, 40), but such outbreaks have not reached pandemic distributions.

Pathogenic *V. cholerae* strains appear to have evolved from environmental, nonpathogenic strains through the recent acquisition of virulence genes via lateral gene transfer (LGT) events (11), and colonization of the human intestine has been an important stage in the evolution of pathogenic *V. cholerae* (29). However, it has been claimed that *V. cholerae* “represents a paradigm for this process” (11). While new virulence factors may clearly facilitate the colonization of new hosts, the transition from the aquatic milieu to a human host milieu is associated with considerable environmental changes, involving shifts in temperature, salinity, nutrient availability, and pH. Hence, suites of adaptations, including adaptive changes to the bacterium’s quorum-sensing system (QSS), in addition to the acquisition of virulence genes, are likely to be required for effective colonization of new hosts or habitats.

We examined if the reasons why *V. cholerae* strains of serotypes O1 and O139, but not of the other serotypes, have been the main causal agents of cholera pandemics can be traced to the level of DNA sequences of the *V. cholerae* QSS basic regulator *hapR*. The function of the *hapR* locus includes positive regulation of protease production and bioluminescence and negative regulation of biofilm formation and virulence when the cell density becomes sufficiently high (17). *hapR* is particularly significant in the *V. cholerae* cycle of pathogenesis and cholera disease transmission because it plays a key role in

the detachment from hosts (14, 23, 24, 53, 54). Here, we report the presence of strong haplotype structure at the *hapR* locus that correlates with the pathogenicity of *V. cholerae* strains and examine the role of natural selection during the evolution of the “pandemic” *hapR2* haplotype, which is found in *V. cholerae* strains of the O1 and O139 serotypes. Specific objectives were to assess the possibilities that natural selection has promoted fixation of the *hapR2* haplotype in pandemic strains and that the observed *hapR* haplotype structure may be correlated with environmental adaptations.

The analyzed strains originate from a wide range of randomly selected global localities and include approximately equal proportions of pandemic O1 and O139 and nonpandemic non-O1 and non-O139 serotypes of *V. cholerae*. The 37 *hapR* sequences (Table 1) obtained from different sources were used to provide independently generated data for the analysis. In addition, two *luxR* sequences, from *V. harveyi* strains BAA-1116 and HY01, were downloaded from GenBank and used as an outgroup in the phylogenetic analysis.

The results of the presented study, including the detection of three mutations in complete linkage disequilibrium (Fig. 1a and b) and two phylogenetic clades (Fig. 2) and the distribution of polymorphic sites (Table 2), provide strong evidence for the existence of two haplotypes, *hapR1* and *hapR2*, of the QSS regulator *hapR*. The complete linkage disequilibrium revealed by comparative nucleotide sequence analysis includes sites at nucleotide positions 998 and 1120 of the protein-coding region of *hapR* (Fig. 1b) and at position 366 in the promoter (Fig. 1a). This is potentially significant since mutations in promoter regions can profoundly affect the regulation and/or functions of genes without altering the structures of the proteins that they encode. The substitution at position 998 is synonymous, while the position 1120 mutation is nonsynonymous and results in the replacement of valine by alanine (Fig. 1a and b). Of the 37 strains examined, 17 possess the *hapR1* haplotype and 20 carry the *hapR2* haplotype. Additional cleaved amplified polymorphic sequence marker analysis based on the A/G polymorphism at position 998, recognized using the restriction enzyme MaeII, confirmed the presence of exactly one copy of either *hapR1* or *hapR2* in each studied *V. cholerae* strain. Maximum

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TABLE 1. *Vibrio cholerae* isolates used in the analysis and the distribution of two *hapR* haplotypes

<i>V. cholerae</i> isolate	Reference(s)	Source, yr of isolation ^a	Biotype	Serotype(s)	<i>hapR</i> haplotype	GenBank accession no.
3083	14, 36	Vietnam, 1964	El Tor	O1 Ogawa	<i>hapR2</i>	AF000716
HK1	9, 23	Hong Kong, 1961 (7th pandemic)	El Tor	O1	<i>hapR2</i>	EU523724
C6706	9, 19, 23, 31	Peru, 1991 (7th pandemic)	El Tor	O1 Inaba	<i>hapR2</i>	EU523710
A1552S	32, 52	Latin America, 1992	El Tor	O1 Inaba	<i>hapR2</i>	EU523709
A1552R	32, 52	Latin America, 1992	El Tor	O1 Inaba	<i>hapR2</i>	EU523708
TSI-4S	48	Not available	El Tor	O1 Ogawa	<i>hapR2</i> (frameshift and terminal deletion)	EU523704
TSI-4R	48	Not available	El Tor	O1 Ogawa	<i>hapR2</i> (frameshift and terminal deletion)	EU523705
P27459	16, 37	Bangladesh, 1976	El Tor	O1 Inaba	<i>hapR2</i>	EU523707
2740-80	9, 23, 36	U.S. Gulf Coast, 1980 (environmental)	El Tor	O1	<i>hapR2</i>	NZ_AAUT0100007
NCTC8457	9, 23	Saudi Arabia, 1910	El Tor	O1	<i>hapR2</i>	NZ_AAWD01000146
MAK757	9, 23, 34	Celebes Islands, 1937 (prepandemic)	El Tor	O1 Ogawa	<i>hapR2</i> (frameshift)	NZ_AAUS0100006
B33	12	Mozambique, 2004	El Tor	O1	<i>hapR2</i>	NZ_AAWO01000108
CB1	This study	Bangladesh, 1982	Classical	O1 Inaba	<i>hapR2</i>	EU523706
569b	7, 36, 39, 47	India, 1948 (clinical)	Classical	O1 Inaba	<i>hapR2</i>	EU523721
CA401	13, 18, 23	India, 1953 (clinical)	Classical	O1	<i>hapR2</i>	EU523726
NIH41	3, 9	India, 1940	Classical	O1 Ogawa	<i>hapR2</i>	EU523723
SG21	15, 23	India, 1992		O139	<i>hapR2</i> (terminal deletion)	EU523725
MO10	9, 23, 49	India, 1992		O139	<i>hapR2</i>	NZ_AAKF0200006
V52	6, 36	Sudan, 1968 (epidemic)		O37	<i>hapR2</i> (terminal deletion)	NZ_AAKJ0200011
V2		India		O37	<i>hapR2</i>	DQ379712
93Ag72	This study	Argentina, 1993 (environmental)		Non-O1, non-O139	<i>hapR1</i>	EU523714
93Ag76	This study	Argentina, 1993 (environmental)		Non-O1, non-O139	<i>hapR1</i> (terminal deletion)	EU523715
NAG V6	This study	Thailand, 1985 (environmental)		Non-O1, non-O139	<i>hapR1</i>	EU523716
NAG V17	This study	Thailand, 1985 (environmental)		Non-O1, non-O139	<i>hapR1</i>	EU523703
NAG V24	This study	Thailand, 1985 (environmental)		Non-O1, non-O139	<i>hapR1</i>	EU523713
NAG V13	This study	Thailand, 1985 (environmental)		Non-O1, non-O139	<i>hapR1</i>	EU523711
V7	This study	Sweden, 2004 (environmental)		Non-O1, non-O139	<i>hapR1</i>	EU523717
V10	This study	Sweden, 2004 (environmental)		Non-O1, non-O139	<i>hapR1</i>	EU523718
V5	This study	Sweden, 2004		Non-O1, non-O139	<i>hapR1</i>	EU523712
93Ag13	46	Argentina, 1993		O8	<i>hapR1</i>	EU523702
93Ag19	This study	Argentina, 1993 (environmental)		O14	<i>hapR1</i>	EU523719
AM-19226	10	Bangladesh, 2001		O39	<i>hapR1</i>	NZ_AATY01000019
1587	8	Peru, 1994		O12	<i>hapR1</i>	NZ_AAUR01000077
PL91	38, 45	India, 1998		O110	<i>hapR1</i>	DQ112157
623-39		Not available			<i>hapR1</i>	NZ_AAWG01000062
MZO-3	10	Bangladesh, 2001		O37	<i>hapR1</i>	NZ_AAUU01000096
SG38-2	15, 23	India, 1992		O139	<i>hapR1</i>	EU523720

^a If the environmental source of a strain is not indicated, the strain was obtained from a patient.

parsimony and maximum likelihood phylogenetic analyses using Mega 4.0 (<http://www.megasoftware.net>) and PAML3.14 packages (51; <http://abacus.gene.ucl.ac.uk/software/paml.html>) indicated that the *hapR1* and *hapR2* sequences group in two separate clades but that *luxR* sequences branch outside the *hapR* clades. In addition, bootstrap values of the maximum parsimony tree provide strong support for the branching node between the two *hapR* haplotype clades (Fig. 2). Estimates of nucleotide diversity per site from the total number of segregating sites (Watterson's θ [50]), average pairwise heterozygosity (π), and a summary of the frequency spectrum of segregating mutations (Tajima's D) (42) were obtained from the sequence fragments using a computer program written in C++ based on the publicly available C++ class library libsequence (44). The program maxhap (<http://home.uchicago.edu/~rhudson1/source>) was used to calculate the scaled recombination rate ($4Nr$ [where N is the effective population size and r is the per-site recombination rate]) from the sequence data

using the composite likelihood method of Hudson (20). The *hapR1* haplotype has a level of polymorphism more than three times higher than that of the *hapR2* haplotype, while a greater excess of singleton sites is observed in the *hapR2* haplotype (Table 2). The coalescent simulations showed that the probability of observing eight segregating sites in a sample of 12 sequences, with 36 segregating sites in the total sample of 27 sequences, is low ($P < 0.0033$). By the four-gamete test (22), five putative recombination events were detected in the *hapR1* haplotype, but none were detected in the *hapR2* haplotype.

The observed polymorphism pattern for the *hapR2* haplotype provides a poor fit to neutral expectations and has all the characteristic hallmarks of a selective sweep accompanying the fixation of beneficial mutations (1, 43): low intrahaplotype polymorphism (Table 2), high linkage disequilibrium, and an excess of low-frequency mutations (Fig. 1a and b). The calculated composite likelihoods of complete sweeps (21, 27, 34) for the two *hapR* clades separately (composite likelihoods of com-

TABLE 2. Polymorphism at the *hapR* locus^a

Haplotype	<i>n</i>	Position	S	S1	Hd	<i>Q</i> _{Wall}	Watterson's θ	π	Tajima's <i>D</i>	Min rec
<i>hapR</i>	27	1151	36	21	0.954	0.056	0.0081	0.0049	−1.468	5
<i>hapR1</i>	15	1151	25	14	0.990	0.080	0.0067	0.0049	−1.110	5
<i>hapR2</i>	12	1151	8	7	0.773	0.250	0.0023	0.0013	−1.778	0

^a *n*, number of sequences; S, number of polymorphic sites; S1, number of singletons; Hd, haplotype diversity (including singletons); *Q*_{Wall}, linkage disequilibrium; π , nucleotide diversity; min rec, minimum number of recombination events detected by the four-gamete test (29).

lotype, while the non-O1, non-O139 strains V52 and V2 of serogroup O37 carry the *hapR2* haplotype (Table 1). As has been discussed, the LGT allows any strain of *V. cholerae* to become a cholera agent (2) and may have promoted the emergence of new virulent *V. cholerae* strains (4, 5, 15). Conditions required for the activation of their transformation competence include high cell density, nutrient limitations, lower water temperatures, and reductions in growth rate and stress (26, 33), all of which can occur in aquatic environments. Moreover, *V. cholerae* cells become naturally competent during growth on the chitin of crustacean exoskeletons (33, 35).

While virulence genes are known to be subject to considerable change (9, 36, 41), we found that the *hapR2* haplotype structure is apparently stable in all known putative stages of the evolution of pandemic *V. cholerae* strains, including classical O1, O1 El Tor, and O139 strains and some representatives of the O37 serogroup (Table 1; also see Table 1 and Fig. 3 in reference 23), conferring a continuous selective advantage. The strong association of *hapR2* with pandemic serotypes could be used as a molecular marker for estimating the epidemic and pandemic potential of a strain. The evolution of the *hapR2* haplotype in pandemic strains appears to have been a significant step in the transition from environmental to pandemic clones and may be involved in cholera disease transmission, since the key role of *hapR* is in the detachment from hosts (14, 23, 24, 53, 54). However, it is possible that *hapR* was not the only locus involved in the environmental adaptation of *V. cholerae*. The extent of the effects of this putative transition in the *V. cholerae* genome, the possibility that changes outside the QSS may have been involved in the divergence of environmental and pathogenic strains, and examination of the way the described allelic diversity contributes to host adaptation and the environmental persistence of *V. cholerae* await further analysis.

Nucleotide sequence accession numbers. The *hapR* nucleotide sequences deposited in GenBank during the course of this study have accession numbers EU523702 to EU523726.

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