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18. Pieces from striatum of newborn wild-type (n = 9), heterozygote (n = 14), and Nurr1$^{-/-}$ (n = 6) mice, and substantia nigra of newborn wild-type (n = 6), heterozygote (n = 12) and Nurr1$^{-/-}$ mice (n = 5) were used for HPLC analysis [see H. Andersson, J. Luthman, E. Lindqvist, L. Olson, *Neurotoxicology* **16**, 201 (1995)]. Pieces from striatum of adult (8 weeks) wild-type (n = 8) and heterozygote (n = 8) mice were used in parallel experiments.
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22. Tissues from 3 (E10.5), 5 (E11.5), and 11 (newborn) mice were used for in situ hybridization with ^{35}S-labeled oligonucleotides [Å. Dagerlind, K. Friberg, A. J. Bean, T. Hökfelt, *Histochemistry* **98**, 39 (1992); (5)]. Sequences of oligonucleotides are available on request.
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12 December 1996; accepted 20 February 1997

Regulation of Lipid A Modifications by *Salmonella typhimurium* Virulence Genes *phoP-phoQ*

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Bacterial pathogenesis requires proteins that sense host microenvironments and respond by regulating virulence gene transcription. For Salmonellae, one such regulatory system is PhoP-PhoQ, which regulates genes required for intracellular survival and resistance to cationic peptides. Analysis by mass spectrometry revealed that *Salmonella typhimurium* PhoP-PhoQ regulated structural modifications of lipid A, the host signaling portion of lipopolysaccharide (LPS), by the addition of aminoarabinose and 2-hydroxymyristate. Structurally modified lipid A altered LPS-mediated expression of the adhesion molecule E-selectin by endothelial cells and tumor necrosis factor–α expression by adherent monocytes. Thus, altered responses to environmentally induced lipid A structural modifications may represent a mechanism for bacteria to gain advantage within host tissues.

Pathogenic bacteria coordinately express virulence genes in response to eukaryotic microenvironments (*1*). For many pathogens, this requires sensing and transcriptional activation involving two proteins that form a phosphorelay mechanism. In Salmonellae, one such system comprises a sensor kinase, PhoQ, and a transcriptional activator, PhoP (*2*, *3*). This system can simultaneously activate and repress more than 40 different genes, termed PhoP-activated (*pag*) and PhoP-repressed (*prg*) genes. The *pho-24* allele, as a result of the replacement of amino acid 48 of PhoQ with isoleucine, locks *S. typhimurium* in a state of *pag* activation and *prg* repression termed the PhoP-constitutive phenotype (PhoP^c) (*4*, *5*). Deletion of *phoP* or *phoQ* results in a PhoP null phenotype (PhoP$^-$) (*2*, *3*). Both PhoP^c and PhoP$^-$ bacteria show decreased virulence, which indicates that the ability to sense various mammalian microenvironments and alter gene transcription is essential for pathogenesis (*2*–*5*). PhoP-PhoQ induces transcription of genes essential to virulence in mice, bacterial survival within macrophages, and resistance to cationic antimicrobial peptides (*2*, *3*, *6*, *7*) and represses genes essential for induction of macropinocytosis in macrophages and epithelial cells (*2*, *3*). Genes in the *pag* group are transcriptionally activated within acidified macrophage phagosomes after *S. typhimurium* phagocytosis by cultured macrophages and after infection of mice as measured by in vivo expression technology (IVET) (*8*, *9*). Therefore, PhoP^c bacteria simulate in part the regulation state of bacteria within host tissues and macrophage phagosomes.

LPS is a pathogenic factor of Gram-negative bacteria that consists of three distinct structural regions: O-antigen, core, and lipid A. Both O-antigen and core consist of polysaccharide chains, whereas lipid A is formed primarily of fatty acid and phosphate substituents bonded to a central glucosamine dimer. Lipid A is the major signaling component of LPS that stimulates cytokine release in the host (*10*).

To investigate whether the PhoP-PhoQ system regulated alteration of lipid A structure, we conducted experiments with lipid A and LPS from various *S. typhimurium* strains (*11*). The fatty acid content of LPS and whole bacteria were studied by gas chromatography (GC) and GC–mass spectrometry (MS). Comparison of the molar ratios of C12:0 versus C14:0 fatty acids (Table 1) showed that the wild-type and PhoP$^-$ strains gave a 1:1 ratio, whereas the PhoP^c strain gave a 2:1 ratio. A previously unreported component of *S. typhimurium* LPS, 2-OH C14:0, was observed in the PhoP^c strain in an amount that would make up for the loss of C14:0 (Table 1). Fatty acid profiles from whole bacteria showed that the PhoP^c strain contained 1.6 nmol of 2-OH C14:0 per milligram of cell dry weight, and the molar ratio of 3-OH C14:0 to 2-OH C14:0 for PhoP^c LPS was similar to that of the whole cell. 2-OH C14:0 was not observed in the whole cell of the wild-type and PhoP$^-$ strains, which indicated that the presence of 2-OH C14:0 in LPS from the PhoP^c strain was not an artifact of LPS isolation. In addition, the total quantity of LPS fatty acid (per milligram of dry weight) indicated that the LPS composition differed among wild-type, PhoP^c, and PhoP$^-$ strains (Table 1), which implied that the LPS in the PhoP^c strain contained less O-antigen polysaccharide relative to the

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lipid A portion of the molecule. Analysis of the LPS carbohydrate profiles confirmed this observation (*12*).

We used MS to study the structural details of lipid A isolated from LPS by SDS-assisted acid hydrolysis (*13*). Because phosphate groups (which readily form anions) were present, all matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) analyses (*14*) and electrospray analyses (*15*) were carried out in negative-ion mode. Although conventional positive-ion electron impact GC-MS (*16*) gave information about the identity of individual side chains, the energetically softer ionization methods were required to examine the intact lipid A. Lipid A from wild-type *S. typhimurium* grown in Luria broth (LB) to logarithmic growth phase and under Mg^{2+}-deficient conditions (*17*), as well as strains with null mutations in *phoP-phoQ* and *pmrA* [a *pag* gene essential for polymyxin resistance (*6*, *7*)], were analyzed by MALDI-TOF MS (Fig. 1). When compared with mass spectra from wild-type lipid A (Fig. 1A), lipid A isolated from the mutants generated different patterns of negatively charged molecular ions. $PhoP^c$ lipid A gave ions that represent the additions of an aminopentose and a hydroxyl group (Fig. 1B). $PhoP^-$ lipid A consisted primarily of hexaacyl lipid A (Fig. 1C), with the depalmitoylated lipid A form predominating. The additional modifications seen with lipid A from $PhoP^c$ were absent. Aminoarabinose (4-amino-4-deoxy-L-arabinose) substitution has been associated with *S. typhimurium* resistance to polymyxin, a cationic peptide (*18*), and is the most likely candidate for the aminopentose modification. PhoP-PhoQ regulates polymyxin resistance by activating the transcription of the *pagB-pmrAB* operon, genes that encode a two-component regulatory system similar in structure to PhoP-PhoQ (*6*, *7*). PhoP-PhoQ–mediated addition

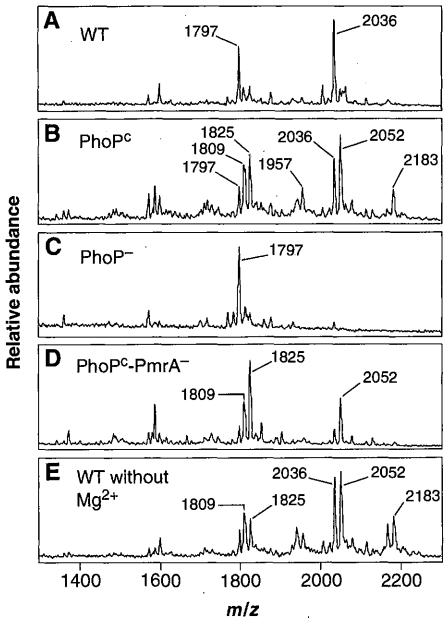

Fig. 1. MALDI-TOF mass spectra showing singly charged anions, $[M-H]^-$, representing the major lipid A structures present after isolation but before any fragmentation in the mass spectrometer. (**A**) Wild-type lipid A showing major signals representing the heptaacylated form of lipid A (*m/z* 2036) and the hexaacylated form lacking palmitate (*m/z* 1797). (**B**) $PhoP^c$ lipid A showing the heptaacylated form with the addition of aminoarabinose and a hydroxyl group (*m/z* 2183) and lacking aminoarabinose (*m/z* 2052). Also shown are the hexaacylated form with aminoarabinose but lacking either 2-OH C14:0 or 3-OH C14:0 (*m/z* 1957), lacking aminoarabinose and either 2-OH C14:0 or 3-OH C14:0 (*m/z* 1825), and lacking aminoarabinose, 2-OH C14:0, or 3-OH C14:0, and a hydroxyl group (*m/z* 1809). (**C**) $PhoP^-$ lipid A showing one major ion at *m/z* 1797, as defined above for the wild type. (**D**) $PhoP^c$-$PmrA^-$ lipid A showing ions at *m/z* 2052, 1825, and 1809 as defined above. (**E**) Wild type grown under Mg^{2+}-deficient conditions, with ions as defined above for $PhoP^c$. Each mass spectrum is a sum of 200 laser shots collected with a PerSeptive Voyager Elite system operated in delayed extraction linear mode. Angiotensin 1 and bovine insulin A-chain were used as internal mass calibrants. Super DHB matrix (5-methoxysalicylic acid/2,5-dihydroxybenzoic acid, 1:10 w/w) was used in a saturated solution of chloroform/methanol, 3:1 v/v.

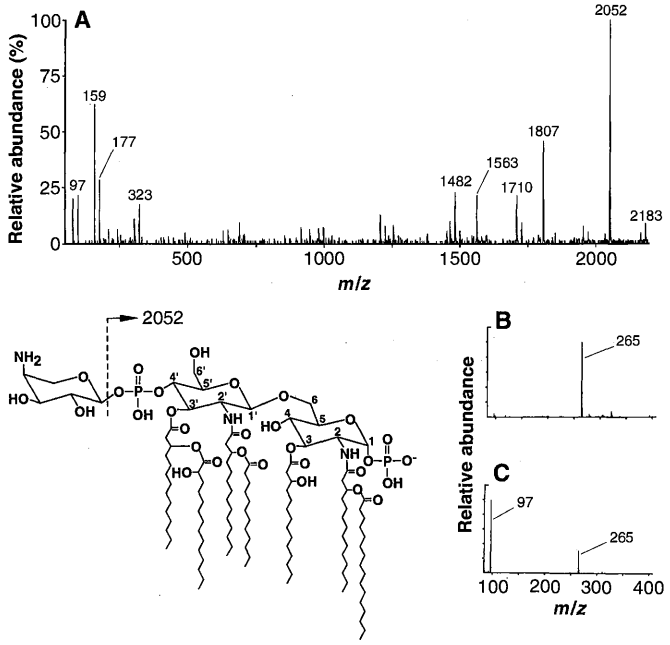

Fig. 2. Chemical structure of $PhoP^c$ lipid A and CAD mass spectra showing its fragmentation. Our structure assignments are based in part on previous nuclear magnetic resonance and MS studies of lipid A from wild-type and mutant strains of *S. typhimurium* (*26*). (**A**) Product ion mass spectrum derived from the $[M-H]^-$ precursor, *m/z* 2183. Key fragments were from neutral loss of aminoarabinose (*m/z* 2052), neutral loss of aminoarabinose + 2-OH or 3-OH C14:0 + H_2O (*m/z* 1807), and aminoarabinose + 2-OH or 3-OH C14:0 + H_2O + H_2PO_4 (*m/z* 1710). Ions at *m/z* 1563 and 1482 represented further neutral losses of hydroxylated C14:0 and H_2PO_4, respectively. The low-mass region contains ions indicating the presence of phosphate (*m/z* 97) or pyrophosphate (*m/z* 159 and 177) and an unknown phosphate-containing fragment at *m/z* 323. The ions at *m/z* 159 and 177 were observed in all diphosphorylated lipid A we have analyzed, regardless of source, expected substitution pattern, or the lack of any other evidence for pyrophosphate substitution, as opposed to two phosphate groups at the 1 and 4′ positions. In monophosphorylated lipid A, these two ions were not observed (*27*). Lipid A at a concentration of 170 pmol/μl was infused at 2.5 μl/min in a solution of chloroform/methanol (3:1 v/v), 0.002% triethylamine by volume, into a Sciex API III+ triple-quadrupole mass spectrometer with a collision energy of 50 eV. The precursor ion *m/z* is selected in the first mass filter, the precursor is fragmented in the second mass filter (which contains argon gas at a pressure of $\sim 1 \times 10^{-3}$ torr), and the product ions are analyzed in the third mass filter (*19*). (**B**) Fragmentation of *m/z* 323 (MS^3) after isolation in the ion trap (*20*). In addition to the neutral loss of 58 mass units shown at *m/z* 265, neutral losses of 18 (H_2O), 28 (CO), and 44 (CO_2) mass units were also observed. (**C**) Fragmentation of *m/z* 265 (MS^4) after isolation from other ions in the spectrum of *m/z* 323, showing the loss of a phosphate group. A Finnigan LCQ electrospray ion trap system was used with infusion conditions similar to those described above for the triple-quadrupole instrument (electrospray source voltage, 3970 V; capillary voltage, 35 V; capillary temperature, 150°C).

Table 1. Fatty acid composition of *S. typhimurium* LPS. LPS fatty acids were analyzed as their methyl esters by capillary GC with flame ionization detection (GC-FID) as described (*22*). The identities of the individual fatty acyl chains were confirmed by capillary GC with electron impact MS. Data shown are the average of three separate analyses (mean ± SD, $n = 3$). ND, not detected.

Fatty acid	LPS (nmol/mg)		
	WT strain	$PhoP^c$ strain	$PhoP^-$ strain
C12:0	46 ± 8	81 ± 9	37 ± 1
C14:0	43 ± 6	45 ± 5	36 ± 3
2-OH C14:0	1.0 ± 2	48 ± 1	ND
3-OH C14:0	130 ± 20	236 ± 25	98 ± 7
C16:0	57 ± 10	118 ± 21	36 ± 8
Total	281 ± 42	532 ± 52	211 ± 5

of aminoarabinose to lipid A was accomplished by promoting the transcription of *pmrAB* because the deletion of this gene eliminated the aminoarabinose modification: Lipid A from the $PhoP^c$-$PmrA^-$ mutant differed qualitatively from the $PhoP^c$ strain only in the absence of aminoarabinose (Fig. 1D). Lipid A from wild-type *S. typhimurium* grown in low Mg^{2+}, a condition that activates PhoP-PhoQ (*17*), was similar to $PhoP^c$ lipid A (Fig. 1E).

Structural interpretations based on the MALDI data (Fig. 1) were confirmed by collision-activated dissociation (CAD) analysis (*19*) using electrospray ionization with the triple-quadrupole mass spectrometer. A CAD spectrum, derived from fragmentation of the heptaacylated lipid A from $PhoP^c$, with a mass/charge ratio (m/z) of 2183, is shown in Fig. 2A. Interpretation of the most abundant product ions was straightforward, with the exception of a signal at m/z 323 observed in all triple-quadrupole CAD spectra (*20*) generated from lipid A containing the 2-OH C14:0 and aminoarabinose modifications. This m/z value was not consistent with the fragmentation of 2-OH C14:0, aminoarabinose, or other known structural features, and suggested the presence of an additional anionic substituent. We used higher order stages of isolation and fragmentation [MS^n (MS to the *n*th power)] in the quadrupole ion trap (*20*) to probe the structure of this ion (Fig. 2, B and C). The trap data confirmed the presence of monophosphate in the unknown structure (or structures). However, our triple-quadrupole MS (Fig. 2A) and MALDI-TOF MS (Fig. 1) results were consistent with a diphosphorylated form of lipid A, not the additional phosphorylation implied by the ion trap studies. An alternative explanation, which is consistent with the other data, is that the presence of the hydroxymyristate or aminoarabinose modifications directs the fragmentation of the molecule such that m/z 323 contains the 4′ phosphate common to all strains analyzed (*21*).

To investigate whether the observed lipid A modifications altered the LPS-mediated host response, we measured the ability of different *S. typhimurium* PhoP-PhoQ mutants to stimulate E-selectin expression in cultured human umbilical cord endothelial cells (HUVEC). E-selectin, an outer membrane adhesion molecule whose expression is induced in response to lipid A (*22, 23*), is also sensitive to changes in the degree of lipid A acylation (*22*). The $PhoP^c$ strain stimulated E-selectin expression less than did wild-type bacteria, whereas the $PhoP^-$ strain generated more stimulation (Fig. 3A). The amount of E-selectin expression stimulated by 2×10^5 wild-type bacteria required 2×10^6 bacteria from the $PhoP^c$ strain (Fig. 3A). Because LPS bioavailability from whole bacteria is a complex process involving multiple factors, purified LPS was tested for the induction of E-selectin by HUVEC. E-selectin stimulation by purified LPS was similarly altered (Fig. 3B). LPS-induced tumor necrosis factor–α (TNF-α) expression by adherent monocytes was also examined (Fig. 3B). Similar to E-selectin stimulation, TNF-α expression per lipid A molecule upon exposure of monocytes to *S. typhimurium* LPS was altered by PhoP-PhoQ regulation; $PhoP^c$ LPS and $PhoP^-$ LPS gave less and more expression, respectively, relative to the wild type (Fig. 3B). Lipid A acylation, and specifically the presence of a myristoyl group, has been identified as an essential element for lipid A signaling in the same assays used above (*22*). Therefore, it is intriguing to speculate that PhoP-PhoQ–regulated lipid A hydroxylation of the myristoyl group altered the host response.

The LPS fatty acid profiles (Table 1) indicate that ~50% of lipid A from the $PhoP^c$ strain contains 2-OH C14:0. The reductions in cell signaling as a consequence of PhoP-PhoQ activation are expected to be larger if bacteria or LPS with a higher percentage of modified lipid A are used in the E-selectin assay. An increase in the modification of lipid A, greater than that seen in the $PhoP^c$ mutant, is likely to occur in vivo because the extent of gene expression activated by the mutation in $PhoP^c$ bacteria is an order of magnitude less than that observed when wild-type bacteria are phagocytosed by macrophages (*8*).

Our studies demonstrate that *S. typhimurium* lipid A is a dynamic structure that is modified in different environments. Therefore, LPS-combating strategies that are based on lipid A structure after in vitro growth may not be appropriate for control of sepsis in vivo. The human immune system may also specifically recognize in vivo modified lipid A. The recent discovery of CD1-positive lymphocytes that recognize the *Mycobacterium tuberculosis* major surface glycolipid lipoarabinomannan (*24*), which is structurally similar to lipid A, raises the possibility that similar human lymphocytes exist that recognize some form of lipid A.

Our observations suggest two mechanisms by which bacteria can combat the host immune system and cause chronic illness. First, LPS modifications may promote resistance to cationic antimicrobial peptides. The aminoarabinose modification is associated with resistance to polymyxin, and it remains to be determined whether PhoP-PhoQ–regulated resistance to defensins (*25*) is in part a result of other modifications of lipid A. Second, host-adapted lipid A may promote bacterial survival by lowering cytokine and chemokine production. Further definition of the mechanism of lipid A modifications will help to clarify their role in bacterial pathogenesis.

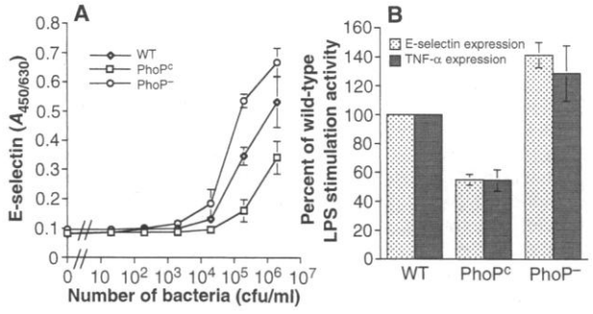

Fig. 3. Alteration in bacterial and LPS-mediated E-selectin expression by HUVEC and TNF-α expression by adherent monocytes as a result of PhoP regulation. Experiments were performed as described (*22*). (**A**) E-selectin expression induced by viable whole bacteria. Different *S. typhimurium* strains were grown in LB to mid-log phase. The cells were adjusted to 2×10^8 cfu/ml (cfu, colony-forming units) by dilution in LB, then diluted with stimulation media (mean ± SD, $n = 3$, representative of at least three separate experiments). (**B**) E-selectin and TNF-α expression induced by purified LPS. HUVEC (1.5×10^4) were stimulated with LPS (100 ng/ml) in a total volume of 100 μl for 4 hours for E-selectin expression; adherent monocytes (2×10^5) were stimulated with LPS (1 ng/ml) in a total volume of 500 μl for 24 hours. $PhoP^c$ and $PhoP^-$ LPS stimulation activities are shown as a percentage of wild-type activity (mean ± SD, $n = 7$ for E-selectin expression pooled from three separate experiments, $n = 8$ for TNF-α expression pooled from four separate experiments). The stimulation activities were calculated as for LPS 3-OH C14:0 content to compare equal molar concentrations of lipid A.

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11. Bacterial strains used in this study include: wild-type, ATCC14028s; PhoP^c, CS022 with *pho*-24 (*4*); PhoP$^-$, CS015 with *phoP*::Tn*10d*-Cam (*3*); and PhoP^c-PmrA$^-$, JSG430 with *pho*-24 *pmrA*::Tn*10d*-Tet (*7*). The Mg^{2+}-ethanol precipitation procedure for isolation of LPS was chosen because it is effective in extracting both smooth and rough LPS with a high degree of purity [R. P. Darveau and R. E. Hancock, *J. Bacteriol.* **155**, 831 (1983)].
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28. We thank K. A. Walsh and L. H. Ericsson for MALDI-TOF and triple-quadrupole mass spectrometers; J. R. Yates III for the ion trap; M. Sanders and W. Loyd for assistance with the ion trap experiments; W. N. Howald for the GC-MS analyses; F. Turecek and W. L. Nelson for reviewing the MS results; M. Gelb for suggesting the synthesis scheme in (*21*); and J. Kowalak, H. Wang, J. Somerville, J. Eng, A. R. Dongre, and E. Carmack for their assistance. Supported by NIH grant R01 AI30479 (S.I.M.) and the School of Pharmacy and Department of Medicinal Chemistry, University of Washington (M.H.).

21 November 1996; accepted 20 February 1997

Insects on Plants: Macroevolutionary Chemical Trends in Host Use

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Determining the macroevolutionary importance of plant chemistry on herbivore host shifts is critical to understanding the evolution of insect-plant interactions. Molecular phylogenies of the ancient and speciose *Blepharida* (Coleoptera)–*Bursera* (Burseraceae) system were reconstructed and terpenoid chemical profiles for the plant species obtained. Statistical analyses show that the historical patterns of host shifts strongly correspond to the patterns of host chemical similarity, indicating that plant chemistry has played a significant role in the evolution of host shifts by phytophagous insects.

What factors have directed the evolution of host shifts by phytophagous insects? This has been a central question in the field of plant-insect interactions for the last 30 years (*1*). Ehrlich and Raven (*2*) postulated that shifts to new hosts are mediated by the chemical similarity between old and new hosts and that host plant chemistry should leave its trace on phylogenetic patterns of host shifts at a macroevolutionary level. However, demonstrating a role for plant chemistry in the macroevolution of host use has been difficult (*3*). Detailed quantitative investigations have had to await the development of modern molecular and phylogenetic techniques to reconstruct accurate host and herbivore trees. Also, an evolutionary association of host shifts with plant chemistry could be spurious: Related plants have similar chemistry, and plant and herbivore phylogenies may correspond for a variety of biogeographic or ecological reasons unrelated to chemistry. In fact, some studies have shown a close correspondence of host and insect phylogenies (*4*, *5*), suggesting that the pattern of host cladogenesis may be important, and that host chemical similarity may be overemphasized. Here, a quantitative investigation of the chemical trace in the evolution of insects and their

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