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Spontaneous phase variation in *Bordetella pertussis* is a multistep non-random process

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Pathogenic strains of *Bordetella pertussis* undergo spontaneous phase variation and become non-pathogenic upon culturing *in vitro*. Spontaneous variants of the Tohama and #165 pathogenic strains of *B. pertussis* were selected by their ability to grow on synthetic and semi-synthetic solid media. The frequency of these variants was between 10^{-6} and 10^{-7}. About 250 variant strains were screened for the presence of virulence-associated traits, such as production of hemolysin, pertussis toxin and filamentous hemagglutinin (FHA). Only four different combinations of the traits were found: 7-11% of the variants displayed all traits, 17% of the variants carried the toxin and FHA, 5-11% carried FHA only and 66% were devoid of all virulence traits. The strains which had at least one virulence trait also demonstrated some adenylate cyclase activity. The disappearance of hemolysin quantitatively affected the other traits. These results suggest that phase variation in *B. pertussis* is a non-random process, involving multistep disappearance of virulence factors in the following order: hemolysin, pertussis toxin and FHA. In contrast, all 300 variants of strain #18323 of *B. pertussis*, which were able to grow on the selective solid media, carried all the virulence traits. This is in accordance with the strain's unique intracerebral growth capability.

Key words: Bordetella pertussis/phase variation/virulence factors/pertussis toxin/adenylate cyclase

Introduction

Pathogenic microorganisms carry virulence factors which enable them to enter the hostile environment, establish an infection in the target tissue and propagate in the presence of the host's defense mechanisms. When grown *in vitro*, the organism is freed from all the host's selective pressures. For the pathogen, continued synthesis and maintenance of the virulence factors becomes an unnecessary metabolic burden. Some pathogens have evolved genetic and physiological mechanisms that facilitate quick adaptation to a completely different habitat (Elwell and Shipley, 1980; Simon *et al.*, 1980). This process, sometimes termed variation, involves various mechanisms depending on the particular organism.

Fresh clinical isolates of *Bordetella pertussis*, the etiologic agent of human whooping cough, display a variety of virulence factors and specific surface antigens such as hemolysins, filamentous hemagglutinin (FHA), dermonecrotic toxin, extracellular adenylate cyclase and a potent exotoxin, which has been variously named: lymphocytosis promoting factor (LPF), histamine sensitization factor (HSF), islet activating protein (IAP), pertussigen and pertussis toxin (for review, see Munoz and Bergman, 1977, 1979). Pertussis toxin is thought to be responsible for many of the systemic effects accompanying *B. pertussis* infections. The wild-type organism is a fastidious grower *in vitro*. Upon *in vitro* passaging, it tends to lose the determinants of pathogenicity, along with some surface agglutinogens, yielding a non-pathogenic variant, with an improved ability to grow on a variety of semi-synthetic and synthetic media (Kasuga *et al.*, 1954). The process of change in *B. pertussis* cultures was called 'phase variation' by Leslie and Gardner (1931) who studied the changes in the antigenic make-up of the bacterium. Thus, the pathogenic wild-type was termed 'phase I' and the fully degraded, non-pathogenic variant was termed 'phase IV' (Leslie and Gardner, 1931) or 'phase III' (Kasuga *et al.*, 1954). Since this process occurs at a relatively high frequency of $10^{-5}-10^{-6}$ (Peppler, 1982) it seems likely that it is more complicated than a simple mutational event (Kleckner, 1977).

Early studies of the variation process claimed that it involves a gradual and sometimes random accumulation of events. Standfast (1951, 1958) noted that changes in virulence for mice and changes in growth requirements may be independent of each other, and suggested that the individual isolates differ in the time required for variation to occur. He also proposed that variants may have already been present in the original clinical isolate. Based on these observations, Parker (1979) suggested that phase variation in *B. pertussis* is a result of a random accumulation of independent mutations.

Since most early studies compared freshly isolated organisms with laboratory passaged variants originating from different clinical isolates, the results are inadequate for genetic analysis of the process. Recently, Peppler (1982) studied isogenic variants of *B. pertussis* and found that a single selective measure resulted in organisms resembling the classical 'phase IV', inasmuch as colony shape, hemolysis and surface antigens were concerned. Thus Peppler's observations disagree with earlier assumptions in claiming that the changes, which comprise the variation process, were dependent on each other and occur in a single step (Peppler, 1982).

We have isolated a number of spontaneous phenotypic variants derived from three pathogenic strains of *B. pertussis* and characterized some of their virulence factors. Based on our observations, 'phase variation' is an ordered gradual process, composed of several non-random and inter-dependent events.

Results

Selection of spontaneous phenotypic variants of B. pertussis

Phenotypic variants of *B. pertussis*, cloned from single, hemolytic colony isolates of the phase I strains Tohama, #165 and #18323, were selected by growth on synthetic and semisynthetic solid media. After 5-10 days incubation at 37°C, colonies appeared on both selective media. Table I gives the frequency of growth on selective media for the three strains of *B. pertussis*. The frequencies for the Tohama I and the #165 strains on both selective media were similar ($10^{-6}-10^{-7}$). The frequency of variants of the #18323

Table I. Frequency of variants growing in defined medium in strains of *B. pertussis*

Strain	Medium	Exp #	CFU x 10^8 on BG agar[a]	Average CFU on selective medium[b]	Variant frequency
Tohama	KED	1	1.4	21 ± 15	1.5×10^{-7}
		2	2.8	109 ± 32	3.9×10^{-7}
	SSA	1	2.6	321 ± 130	1.2×10^{-6}
		2	5.2	590 ± 56	1.1×10^{-6}
#165	KED	1	1.8	342 ± 100	1.9×10^{-6}
		2	5.4	291 ± 72	5.4×10^{-7}
	SSA	1	3.0	200 ± 50	6.7×10^{-7}
#18323	KED	1	4.0	10 ± 6	2.5×10^{-8}
		2	3.4	164 ± 37	4.8×10^{-7}
	SSA	1	10.2	91 ± 22	8.9×10^{-8}
		2	6.0	44 ± 9	7.2×10^{-8}

[a]Number of colony forming units (CFU) in 0.1 ml of bacterial suspension.
[b]Number of colony forming units (CFU) ± standard deviation in 0.1 ml of bacterial suspension.

Table II. Gross analysis of traits in phenotypic variants of *B. pertussis*

Group	Traits present[a]			Frequency		
	Hemolysis	Toxin	FHA	in Tohama	in #165	in #18323
A	*	*	*	11%	7%	100%
B		*	*	17%	16%	–
C			*	5%	11%	–
D				67%	66%	–

[a]Traits were determined on BGA grown colonies by direct visualization for hemolysis and by *in situ* solid phase enzyme immunoassay for pertussis toxin and for FHA.

Table III. Frequency of virulence traits in the sample of *B. pertussis* spontaneous variants

Trait[a]	Frequency in	
	Tohama	#165
Hemolysis	10.9%	6.6%
Toxin	28.0%	23.1%
FHA	33.4%	33.7%

[a]See footnote a for Table II.

strain was lower ($10^{-7}-10^{-8}$). The single variant colonies thus obtained were expanded by growth on non-selective Bordet-Gengou (BG) blood agar plates and stored as described in the Materials and methods section for further characterization.

Characterization of selected B. pertussis phenotypic variants

110 variants derived from Tohama phase I strain of *B. pertussis*, 150 variants derived from strain #165 and 300 variants from strain #18323 were initially screened for three prominent virulence factors: hemolysis in BG agar, production of antigenic pertussis toxin and production of antigenic filamentous hemagglutinin (FHA). Only four different combinations were found in the progeny of Tohama and #165 strains. Table II summarizes the characteristics of the four groups of variants and their frequencies within the tested sample. Group A variants carry all three virulence factors, which we screened for. Thus, members of group A differ from wild-type (or phase I) organisms only in their ability to grow on defined solid media. Variants in group B do not show the characteristic zone of hemolysis around their colonies, but they synthesize pertussis toxin and FHA. Group C variants display only the FHA and group D do not carry any of the traits in question. All variants of strain #18323 belonged to group A.

Table III shows the calculated frequencies of individual traits in the sample of variants. It appears that the frequencies are similar to both Tohama and #165 strains. The frequency is highest for FHA, followed by the pertussis toxin and hemolysin. From this data we infer that the hemolysis trait is the first to disappear from the population, the toxin is the second and the FHA is the most stable of the traits we examined.

Pathogenic *B. pertussis* strains produce surface and soluble adenylate cyclase, which is similar to the eukaryotic enzyme in its dependence on calmodulin (Wolff *et al.*, 1980). Its exact role in pathogenesis is not yet completely clear (Confer and Eaton, 1982). The activity of calmodulin-dependent adenylate cyclase was assayed in all the variants relative to the known virulent traits. Analysis of the phenotypes within the four groups in relation to the cyclase activity appears in Table IV. The relative amounts of pertussis toxin and FHA, as far as could be judged from the results of the qualitative immunoassay we have employed, are also presented in this table. It appears that the cyclase trait is mainly linked to the hemolytic activity of the variant, since there is at least a 10-fold decrease in cyclase activity in group B as compared to

Table IV. Fine analysis of *B. pertussis* variants within phenotypic groups and their correlation with adenylate cyclase activity

Group	Phenotype[a]			#165		Tohama	
	hemolysis	toxin	FHA	cyclase[b]	frequency	cyclase[b]	frequency
A	+ + +	+ + +	+ + +	1530 ± 880	7%	665 ± 163	11%
B1	–	+ +	+ +	46 ± 38	7%	16.5 ± 18.2	6%
B2	–	+	+ +	0.2 ± 0.4	5%	3.1 ± 4.1	6%
B3	–	+ –	+ +	0	4%	0.5 ± 0.1	5%
C	–	–	+ +	0.5 ± 1.0	11%	1.1 ± 1.8	5%
D	–	–	–	0	66%	0	67%
wild-type	+ + +	+ + +	+ + +	800		750	

[a]See footnote a for Table II.
+ + + = level similar to the wild-type;
+ +, +, + – = level reduced compared to wild-type;
– = negative for that trait.
[b]Adenylate cyclase activity is expressed in μmol/15 min/mg protein.

group A or the wild-type strain. The remaining cyclase activity in the other variants is linked to the pertussis toxin trait, where it decreases in concert with the decreasing amount of toxin in the group B variants. It should be noted here, that group D variants resemble the classical phase III (Kasuga *et al.*, 1954) or phase IV (Leslie and Gardner, 1931) variants of *B. pertussis* in their complete lack of hemolysis, pertussis toxin, adenylate cyclase and FHA.

Discussion

Phase variation of *B. pertussis* has been known for over 50 years (Leslie and Gardner, 1931), but only in recent years have the distinct components of the bacterium been identified, isolated and their functions elucidated. Pertussis toxin and the FHA are important in the pathogenesis and acquired protection against the disease (Cowell *et al.*, 1982). The calmodulin-dependent adenylate cyclase may also prove to be an important virulence factor (Confer and Eaton, 1982).

Peppler (1982) described a new approach for the study of phase variation in *B. pertussis* by isolating variants from single colony isolates of pathogenic strains. We have combined the approach of Peppler (1982) with the technology developed by Cowell *et al.* (1982) for the isolation and identification of the toxin and FHA of *B. pertussis* in an attempt to devise a rational approach for the study of spontaneous phase variation in this organism. The selection procedure we have employed identified four different phenotypes in the selected population, only one of them (group D, Table II) resembling the classical phase III (Kasuga *et al.*, 1954) or phase IV (Leslie and Gardner, 1931). The other three phenotypes were intermediates between the wild-type and the fully degraded strain and carried all (group A, Table II) or part (groups B and C, Table II) of the virulence traits we screened for. Our results suggest that spontaneous phase variation is a multistep process, involving intermediate phenotypes between the wild-type (the classical phase I) and the fully degraded, non-virulent phase III or IV phenotype. Our data also show that the variation process is a non-random sequence of interdependent events. One would expect to find eight different combinations of the three traits we have screened, if it were a completely random process; our sample of variants, from both strains, had only four combinations (Table II). Assuming that the frequency of each combination of traits in our sample reflects the stability of that particular phenotype, then the phenotype resembling the fully degraded classical phase III or IV of *B. pertussis* is the most stable – it constitutes 65% of the population. The total sample frequency of each separate trait (Table III) may in fact indicate the order of disappearance from the population. However, all the phenotypes were already present in hemolytic colonies of cloned wild-type strains, growing on the non-selective BG agar medium and they are stable upon passaging *in vitro* for a large number of generations on selective and non-selective media. Thus, variation occurs spontaneously in wild-type colonies and the variation process stops once the variants are isolated and cloned.

The notion that the individual traits are inter-dependent on each other is exemplified by the data in Table IV. In the hemolytic variants the levels of the other traits (toxin, FHA and adenylate cyclase) are equal or similar to the wild-type. Upon disappearance of hemolysis from a variant the amount of all other traits are comparatively reduced. This is especially conspicuous for the activity of adenylate cyclase (Table IV). A similar effect of lack of hemolysis on the level of cyclase was reported by Weiss *et al.* (1983), who studied transposon mutagenesis in *B. pertussis*. Peppler (1982) identified only one phenotype in his selected population, which was devoid of all virulence factors, similar to our group D. Thus his conclusions, that phase variation is a one-step process, do not agree with our observations. The order of disappearance of virulence factors we have observed, is also in contrast to Parker's (1979) theory, that variation is a random accumulation of mutations.

The fixed combination of traits in two distinct strains of *B. pertussis* and the partial polarity of the variation may indicate that the genes controlling the synthesis of virulence factors are arranged in a linear order on the genome or on a movable genetic element. In that case, our data may be explained by an ordered deletion process, similar to the mechanism described for the formation of Lambda gal transducing phage (Campbell, 1971). The existence of an operon, incorporating the virulence factors of the bacterium, is supported by the reversible 'mode conversion' of virulent *B. pertussis* to a non-virulent phenotype when grown in the presence of high concentrations of nicotinic acid (Pusztai and Joo, 1967; McPheat *et al.*, 1983) or magnesium (Lacey, 1960) and by the mutagenesis data of Weiss *et al.* (1983), who obtained a mutant of *B. pertusis*, devoid of all virulence factors by a single transposon mutation. The disappearance of virulence traits may, in addition, be explained by the existence of plasmids or lysogenic phages, each coding for one or more virulence traits. However, such virulence-associated genetic vectors have not been identified so far in *B. pertussis* (Weiss and Falkow, 1982, 1983 and our unpublished results) and more work is required to resolve this problem.

The relevance of phase variation to the ability to cause disease is probably the most interesting facet of the process. Various definitions for virulence exist for *B. pertussis* (e.g., Weiss *et al.*, 1983; Peppler, 1982) because there is no known rational model for the disease in animals. The intracerebral challenge model, which is currently employed in vaccine testing, poses several problems since not all virulent strains of *B. pertussis* are active in this assay (Standfast, 1958). New approaches are being studied (Sato *et al.*, 1980) and these may provide better models for the disease, enabling us to describe virulence more precisely in genetic terms. That variation is detrimental to pathogenicity via the intracerebral route was suggested by Standfast (1958) and supported by our inability to challenge mice intracerebrally with the #165 strain (unpublished data). This is further exemplified by our observations that the #18323 intracerebral challenge strain of *B. pertussis* is resistant to variation, since no loss of virulence traits occurred upon selection on defined and semi-defined media (Table II). Therefore, as far as the intracerebral challenge model for *in vivo* infection is concerned, all virulence factors and inability to undergo variation are prerequisites for pathogenicity.

Materials and methods

Bacterial strains

B. pertussis strains #165, #18323, Tohama phase I and Tohama phase III and *B. bronchiseptica* #477 were obtained from Dr. Charles Manclark, Director of the Pertussis Branch at the Office of Biologics, National Center for Drugs and Biologics, Bethesda, Maryland, USA. The skim milk lyophilized strains were resuspended in phosphate buffered saline (PBS) and grown on Bordet-Gengou (BG) agar base (Difco, Detroit, Michigan, USA) supplemented with 15% v/v fresh defibrinated sheep blood. The bacteria were

cloned and subcultured on BG plates, suspended in a solution of 50% glycerol in PBS and stored at −70°C.

Selective media

Variants were selected on each of the following solid media. The synthetic Stainer and Scholte (1971) medium was solidified with 1% w/v Difco's Bacto Agar (SSA) and the KED semi-synthetic casein hydrolysate agar medium (Kloos *et al.*, 1979) was prepared without activated charcoal.

Antisera and antibody enzyme conjugates

Anti-pertussis toxin (= anti-LPF) and anti-FHA sera were prepared in goats at the Pertussis Branch (USA). The anti-toxin antibody was absorbed with Sepharose (Pharmacia, Uppsala, Sweden) bound FHA, affinity-purified on Sepharose bound pertussis toxin and conjugated with horseradish peroxidase (Boehringer, Mannheim, FRG) by the method of Nakane and Kawasi (1974). Donkey anti-goat IgG antiserum was obtained from Miles-Yeda (Rehovot, Israel), absorbed with *B. bronchiseptica* cultures (Guinee *et al.*, 1976) and conjugated with horseradish peroxidase as above.

Protein determination of bacterial suspensions

The protein of bacterial cell suspensions was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Selection of phenotypic variants

Small individual colonies of phase I strains (#165, Tohama, #18323), showing pronounced hemolysis on BG plates after 3−5 days incubation at 37°C, were suspended in PBS and cultured on fresh BG agar plates for 48 h. Cells were collected with a wire loop and washed twice in PBS. The pellet was resuspended in PBS to an absorbance of 5.0 at 650 nm. This suspension was serially diluted in PBS and 100 microliter aliquots of each two-fold dilution were plated on BG agar, SS agar and KED agar plates (see above). The plates were incubated at 37°C and colony counts were performed after 5 and 10 days. The frequency of variants, capable of growth on defined media, was calculated by comparison with the growth on BG agar. 150−300 individual variant colonies derived from each wild-type strain were passaged once on the same selective medium and then replica plated on the non-selective BG agar for storage and propagation.

In situ screening for FHA-producing colonies

Antigenic FHA on individual colonies was detected by a modification of the enzyme immunosorbent assay for bacterial colonies described by Buckel and Zehelein (1981). Variant replica colonies were grown on BG agar plates for 3−5 days at 37°C. The plates were inverted over a dish containing chloroform for 10 min. High impact polystyrene (Rabi Trading Ltd, Tel Aviv, Israel) 9 cm circles were pressed into the chloroform-treated plates and allowed to incubate for 10 min at room temperature. The circles were then rinsed thoroughly with saline containing 0.1% w/v Brij 35T detergent (Sigma, Petah Tikva, Israel) and immersed in a 1:2000 dilution of a goat anti-FHA antiserum in PBS containing 0.1% w/v Brij 35T and 4% w/v polyethylene glycol (PEG) 6000 (Salonen and Vaheri, 1981). Following a 60-min incubation at 37°C (Salonen and Vaheri, 1981) the circles were rinsed thoroughly in Saline-Brij and were incubated with a 1:2000 dilution of peroxidase-conjugated donkey anti-goat IgG antiserum (see above) in PBS-Brij-PEG. Following a 60-min incubation at 37°C, the circles were washed several times in Saline-Brij and finally in distilled water. Visualization of the FHA-containing colonies was done exactly as described by Buckel and Zehelein (1981).

Screening for pertussis toxin-producing colonies

This was performed essentially as described above for FHA, except for employing a peroxidase-conjugated anti-pertussis toxin antibody and omitting the use of the conjugated anti-goat IgG antibody.

Determination of adenylate cyclase activity

The *B. pertussis* variants were grown from frozen stocks for 3 days on BG agar plates and then transferred to fresh BG plates for an additional 24-h growth period at 37°C. The cells were collected from the agar with a wire loop and suspended in distilled water. The suspension was incubated at 37°C for 15 min and cooled immediately on ice. Adenylate cyclase activity in the suspensions was assayed by the method of Salomon (1979). The reaction was carried out in 50 microliter volumes containing 60 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.3 mM $CaCl_2$ and a 1:50 dilution of crude rabbit calmodulin (a gift from Dr. Y.Solomon, The Weisman Institute of Science). Ten microliter samples of five-fold serial dilutions of the suspensions were added to the reaction mix and incubated for 10 min at 30°C before [^{32}P]ATP was added to a final concentration of 1 mM (0.6−10.0 x 10^6 c.p.m.) to start the reaction. The activity of adenylate cyclase was expressed as micromoles cAMP/15 min/mg bacterial protein.

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