Changes in environmental temperature represent one of the major stresses faced by microorganisms as they affect the function of the cytoplasmic membrane. In this study, we have analyzed the thermal adaptation in two closely related respiratory pathogens Bordetella pertussis and Bordetella bronchiseptica. Although B. pertussis represents a pathogen strictly adapted to the human body temperature, B. bronchiseptica causes infection in a broad range of animals and survives also outside of the host. We applied GC-MS to determine the fatty acids of both Bordetella species grown at different temperatures and analyzed the membrane fluidity by fluorescence anisotropy measurement. In parallel, we also monitored the effect of growth temperature changes on the expression and production of several virulence factors. In response to low temperatures, B. pertussis adapted its fatty acid composition and membrane fluidity to a considerably lesser extent when compared with B. bronchiseptica. Remarkably, B. pertussis maintained the production of virulence factors at 24 °C, whereas B. bronchiseptica cells resumed the production only upon temperature upshift to 37 °C. This growth temperature-associated differential modulation of virulence factor production was linked to the phosphorylation state of transcriptional regulator BvgA. The observed differences in low-temperature adaptation between B. pertussis and B. bronchiseptica may result from selective adaptation of B. pertussis to the human host. We propose that the reduced plasticity of the B. pertussis membranes ensures sustained production of virulence factors at suboptimal temperatures and may play an important role in the transmission of the disease.

Adaptability is one of the crucial features of microorganisms allowing them to endure and thrive within a wide range of environmental conditions. To adapt, bacteria must sense and respond to any alteration of the environment such as nutrient or oxygen limitation, pH and osmotic stresses, and changes in temperature. Change in the ambient temperature is one of the major stresses that bacteria face as it, among other effects, influences the fluidity of their plasma membrane and function of the cell envelope (1). Although the membrane lipid bilayers are mostly fluid at physiological temperatures, at lower temperatures they undergo a reversible transformation from disordered (fluid) to ordered (non-fluid) conformation (1, 2). Suboptimal fluidity of membrane lipids may result in defective membrane functioning; nevertheless, bacterial cells possess the ability to modulate and adjust the membrane fluidity in response to temperature fluctuations (3, 4). To fine-tune the optimal fluidity, bacteria use several mechanisms; however, in Gram-negative bacteria, the predominant response is based on remodeling of phospholipid acyl chains by alteration of the ratio between saturated (SFA) and unsaturated (UFA) fatty acids (4–7). The steric hindrance introduced by double bonds present in UFAs results in less tight acyl chain packing and increased fluidity. Consequently, lipids containing UFAs have lower transition temperatures than those made of SFAs (8). Therefore, bacterial cells respond to a decrease in membrane fluidity resulting from temperature drop by increasing the proportion of UFAs in the phospholipid membrane (7). In conclusion, temperature-dependent regulation of fatty acid synthesis allows cells to maintain the appropriate fluidity of the membrane regardless of the ambient temperature (9, 10).

To persist, bacterial pathogens must adapt to environmental niches both outside and inside of the host. In response to different stimuli encountered upon entry to the host (e.g. increased temperature and lack of nutrients), pathogens produce specific gene products permitting efficient infection and survival within the host. Members of the genus Bordetella are

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3 The abbreviations used are: SFA, saturated fatty acid; UFA, unsaturated fatty acid; ACT, adenylate cyclase toxin; CFA, cyclopropane fatty acid; DI, desaturation index; DPH, 1,6-diphenylhexatriene; FA, fatty acid; FHA, filamentous hemagglutinin; PRN, pertactin; RT-qPCR, quantitative reverse transcription-polymerase chain reaction; AC, adenylate cyclase; FAME, fatty acid methyl ester; BvgA-P, phosphorylated BvgA.
Gram-negative predominantly respiratory pathogens that cause infection in humans and animals. Two closely related species, *Bordetella pertussis* and *Bordetella bronchiseptica*, produce a variety of virulence factors to successfully infect and persist within the host (11, 12). These factors include adhesins, such as filamentous hemagglutinin, fimbriae, and pertactin, and toxins, such as adenylate cyclase toxin. Whereas adhesins ensure attachment of bacterial cells to ciliated epithelial cells in the respiratory tract, macrophages, and neutrophils, toxins promote survival of bacteria by subverting immune responses of the host (13, 14). For example, the enzymatic activities of adenylate cyclase toxin manipulate cAMP signaling in immune cells (14, 15) and thereby cause the inhibition of the chemotactic, phagocytic, and oxidative burst capacities of the host phagocytes (14, 16, 17).

The expression of the majority of *Bordetella* virulence factors is controlled at the transcriptional level by a two-component system encoded by the *bvg* locus (18, 19). It consists of the transmembrane sensor kinase BvgS and the DNA-binding response regulator BvgA, which in its phosphorylated form binds to promoter regions and activates transcription of dependent virulence genes (20, 21). BvgAS phosphorelay controls a whole spectrum of gene expression states transitioning cells between Bvg− phase, intermediate Bvg+ phase, and nonvirulent Bvg− phase (22). The environmental signals sensed by the BvgS kinase are unknown; nevertheless, the activity of the BvgAS system can be modified under laboratory conditions, and this process is called antigenic or phenotypic modulation (23–25). Although cultivation at 37 °C induces BvgAS activity in *Bordetella* cells, temperatures below 25 °C or the presence of millimolar amounts of sulfate or nicotinic acid in media renders the BvgAS system inactive (24, 26).

Although *B. pertussis* and *B. bronchiseptica* express a similar array of virulence genes, they exhibit different host ranges and survive within different environments. Whereas *B. pertussis* represents a pathogen strictly adapted to the human body, *B. bronchiseptica* infects a broad range of animals and survives also outside of the host (27, 28). Comparative analysis of the genome sequences of *B. pertussis* and *B. bronchiseptica* revealed that *B. pertussis* has evolved from a *B. bronchiseptica*-like ancestor by gene inactivation and loss (29, 30). Among those genes that were found exclusively in *B. bronchiseptica* and were probably lost in *B. pertussis* several genes related to cell membrane and fatty acid biosynthesis were identified (29). These data suggest that the inability of *B. pertussis* to survive outside of the host might be linked to altered composition of the membrane.

Thermal adaptation upon entry into the host environment represents one of the crucial factors of successful infection (31). Therefore, in this study, we have compared the ability of both *B. pertussis* and *B. bronchiseptica* cells to acclimate their membranes to different temperatures in vitro. We have followed the hypothesis that, during the adaptation process to a human host, *B. pertussis* may have lost the capacity to adjust its membrane to thermal changes. Simultaneously, we monitored the effect of thermal changes on the expression and production of bvg-dependent virulence factors.

**Results**

*B. bronchiseptica* adjusts its membrane composition to a greater extent compared with *B. pertussis*

First, we have determined membrane phospholipid fatty acid (FA) composition of *B. pertussis* Tohama I and *B. bronchiseptica* RB50 cells grown at 37 °C. Results presented in Fig. 1A show that the composition of *B. pertussis* membrane bilayer is relatively simple, containing predominantly phospholipids esterified with palmitic (16:0), stearic (18:0), and palmitoleic (16:1) acids, whereas the fatty acid composition of *B. bronchiseptica* membrane (Fig. 1B) is more complex as it, in addition, contains cyclopropane fatty acid (CFA; cis-9,10-methylenehexadecanoic acid; Δ17:0) and oleic (18:1) acids. In both strains, the major FA was palmitic acid, comprising more than 50% of the total FAs. Next, we have analyzed the FA content in cells grown at 24 and 16 °C. For both strains, we have calculated the desaturation index as the weighted average of the number of double bonds per fatty acid. As expected, in response to lower temperatures, the content of UFAs increased in both strains, but to strikingly different extents (Fig. 1, A and B). In *B. pertussis*, the content of UFAs at 24 °C increased only by 23% when
Host adaptation affects responsiveness to thermal changes

Figure 2. Growth temperature-dependent changes of DPH fluorescence anisotropy in membrane lipids. A and B, B. pertussis (B. p.) Tohama I (A) and B. bronchiseptica (B. b.) RB50 (B) cells were grown at 37 °C, 24 °C, and 16 °C, and membrane lipids were used to form liposomes. Steady-state fluorescence anisotropy (r_{ss}) of DPH probe embedded into liposomes was measured along the temperature range indicated on the x axis. Error bars represent standard deviations from three measurements and are shown if larger than the symbols. At least two independent experiments were performed, and representative results are shown.

The temperature-induced changes in membrane composition affect the membrane fluidity

Different levels of membrane desaturation observed at lower cultivation temperatures implied that fluidity of the B. bronchiseptica and B. pertussis membranes should also be affected to a different extent. Therefore, membrane fluidity was determined by measurement of steady-state fluorescence anisotropy with 1,6-diphenylhexatriene (DPH). DPH is a lipophilic fluorophore that penetrates into the hydrophobic core of the membrane and orients perpendicularly to the membrane plane (32). Increased fluidity of the membrane allows the DPH probe to rotate to a greater extent, which results in depolarization of the fluorescence emission and a decrease in the DPH fluorescence anisotropy.

In agreement with only a slight change in fatty acid composition, the fluidity of B. pertussis membrane did not change notably in response to low growth temperatures (Fig. 2A). In contrast, the DPH anisotropy values determined in membrane lipids isolated from B. bronchiseptica cells cultivated at 24 and 16 °C markedly decreased when compared with 37 °C, indicating that at both lower temperatures the efficient desaturation of fatty acid (Fig. 1B) resulted in increased membrane fluidity (Fig. 2B). In other words, in contrast to B. pertussis, B. bronchiseptica was competent to adjust the fluidity of the membrane to low temperatures.

Next, to corroborate our observation in more detail, we monitored the thermal adaptation in B. bronchiseptica and B. pertussis in a time-dependent manner. Furthermore, we tested whether and how the observed differences in membrane adaptation correlated with the expression and production of virulence factors.

B. bronchiseptica effectively adapts its membrane composition upon temperature upshift from 24 to 37 °C

Cell cultures of both Bordetella species were grown to exponential phase at 24 °C and then transferred to 37 °C for 6 h. Samples for fatty acid analysis were taken before the shift (T0h) and 1, 2, 4, and 6 h after the shift (T1h–T6h). Sample of cells grown independently at 37 °C served as a control. The analysis of fatty acid composition (Fig. 3B) revealed that B. bronchiseptica cells gradually remodeled the cell membrane during the temperature upshift. As expected, the content of palmitic and cyclopropane SFAs continuously increased, whereas the amounts of palmitoleic and oleic UFAs decreased. Consequently, the DI value dropped from 0.58 to 0.32 within 6 h of cultivation at 37 °C. The index determined in control sample grown at 37 °C (0.27) suggested that after 6 h the RB50 cells were almost fully adapted to higher temperature. In B. pertussis, the desaturation decreased only slightly from 0.50 to 0.43; nevertheless, the DI value obtained in the control sample (0.43) suggested that Tohama I cells also adapted the membrane composition during the upshift (Fig. 3A). Similarly, assessment of the membrane fluidity using DPH anisotropy measurements confirmed efficient membrane adaptation upon temperature upshift.
Host adaptation affects responsiveness to thermal changes

upshift in *B. bronchiseptica* cells (Fig. 3C). As anticipated, the DPH anisotropy values increased progressively upon shift to 37 °C and indicated that fluidity of the membrane had been adjusted to higher growth temperature, whereas in *B. pertussis* samples the fluidity did not change significantly due to only subtle changes in membrane composition (data not shown).

**Differential production of virulence factors in Tohama I and RB50 strains upon temperature shift from 24 to 37 °C**

The expression and production of the majority of virulence factors produced by bordetellae are regulated by the two-component system BvgAS, the activity of which is temperature-dependent. Because BvgS kinase transmits the activation signal through the membrane, we speculated that low temperature-induced changes in membrane composition may influence the Bvg-dependent circuit. To validate our hypothesis, samples of Tohama I and RB50 strains were taken in parallel during the temperature upshift (T0h–T6h) and assayed for production of the adenylate cyclase toxin (ACT), filamentous hemagglutinin (FHA), pertactin (PRN), and BvgA proteins by immunoblotting. Similar to ACT, these proteins were detected in significant amounts in *B. bronchiseptica* membrane lipids upon temperature upshift. Error bars represent standard deviations from three measurements and are shown if larger than the symbols. At least two independent experiments were performed, and representative results are shown.

Western blot analysis of ACT protein levels in *B. pertussis* and *B. bronchiseptica* cells during temperature upshift confirmed that, in contrast to *B. bronchiseptica*, *B. pertussis* produces the toxin at 24 °C, switched on the production of the toxin at 2 h upon shift and reached the maximal production within 6 h after the temperature upshift (Fig. 4B).

Furthermore, the expression of selected virulence genes during the transition from 24 to 37 °C was analyzed by quantitative reverse transcription-polymerase chain reaction (RT-qPCR). The relative levels of *cyaA*, *flaB*, and *bvgA* mRNAs (encoding ACT, FHA, and BvgA proteins, respectively) were analyzed in samples taken before and 2, 4, and 6 h after the shift as well as in
Host adaptation affects responsiveness to thermal changes

Figure 4. Temperature-dependent production of virulence factors. *B. pertussis* (B. p.) Tohama I and *B. bronchiseptica* (B. b.) RB50 strains were cultured in Stainer-Scholte medium at 24 °C. Samples for ACT activity measurements and Western blot analysis were taken before (T0h) and 1, 2, 4, and 6 h (T1h–T6h) after transfer to 37 °C. In parallel, control samples were taken from cultures grown separately at 37 °C (at A600 similar to that of the T6h sample). A and B, culture samples of *B. pertussis* (A) and *B. bronchiseptica* (B) strains were mixed with 8 M urea (1:1) and used to determine the total AC enzyme activity (secreted into the medium plus associated with cells). Error bars represent standard deviations from three measurements. Two independent experiments were performed, and representative results are shown. C and D, Western blot analysis of ACT, FHA, PRN, and BvgA protein levels in samples taken before (lane 1) and after shift to 37 °C (lanes 2–5) and in a control sample (lane 6). Whole-cell lysates of *B. pertussis* (C) and *B. bronchiseptica* (D) cultures equivalent to 0.1 A600 unit were separated by standard SDS-PAGE and transferred to nitrocellulose membrane. Protein levels were analyzed by immunoblotting using corresponding antibodies. Relevant parts of the membranes are shown. At least three independent experiments were performed, and representative results are shown. mU, milliunits.

control samples grown separately at 37 °C. As shown in Fig. 5B, expression levels of all three genes in *B. bronchiseptica* were strongly increased in response to temperature switch. In contrast, in *B. pertussis*, only the expression of *cyaA* was slightly increased (Fig. 5A).

Thermal adaptation of the clinical isolate B1917 is similar to that of Tohama I strain

Collectively, obtained data indicated that the ability to respond to thermal changes was diminished in the Tohama I strain. Tohama I represents a reference strain of *B. pertussis*; however, this laboratory strain was passaged in vitro multiple times and does not represent currently circulating *B. pertussis* strains (29, 33). Therefore, in addition, we examined the well-characterized recent clinical isolate of *B. pertussis*, the B1917 strain (34). As documented in Fig. 6, we have repeated all the experiments with this low-passage strain. Apparently, its membrane composition and its ability to adapt fatty acid content to thermal changes were very similar to those of the Tohama I strain. Membranes of B1917 strain contained comparable amounts of palmitic (16:0), stearic (18:0), and palmitoleic (16:1) fatty acids, and the changes in fatty acid content during the shift from 24 to 37 °C were almost identical to those observed with Tohama I (Fig. 6A). Consequently, the degree of desaturation upon thermal shift decreased from 0.51 to 0.43 and almost copied the values obtained in the Tohama I strain. Interestingly, the expression patterns of studied virulence genes determined by RT-qPCR were different from those observed with the Tohama I strain (Fig. 6B). Particularly, the expression of *cyaA* gene displayed a relatively high increase; however, increased transcription did not translate into significantly increased production of ACT (Fig. 6C). Essentially, the production of virulence factors during the temperature upshift did not differ dramatically from that in the Tohama I strain, and all the analyzed proteins were also detected already before the temperature shift (Fig. 6D).

*B. pertussis* is less sensitive to modulatory effects of low temperature than *B. bronchiseptica*

The sustained production of virulence factors at 24 °C suggested that in both *B. pertussis* strains the BvgAS system is operative even under suboptimal temperatures. To prove this assumption, we have determined the levels of BvgA phosphorylation during the temperature upshift in *B. pertussis* Tohama I and B1917 strains and in *B. bronchiseptica* RB50 strain. Phosphorylated (BvgA-P) and nonphosphorylated BvgA proteins were separated by SDS-PAGE with Phos-tag™ and detected by Western blot analysis. As shown in Fig. 7A, significant amounts of BvgA and BvgA-P proteins were detected in samples of both *B. pertussis* strains grown at 24 °C. Levels of both BvgA species further increased upon temperature upshift. Notably, in *B. bronchiseptica* cells grown at 24 °C, only minute amounts of nonphosphorylated BvgA could be found; however, upon shift to 37 °C, the levels of both BvgA-P and BvgA proteins increased considerably. These results were in line with the results of Western blot analysis and clarified persistent production of virulence factors in *B. pertussis* at low temperature. Nevertheless, the presence of BvgA-P protein at 24 °C was rather unexpected as it was shown that in the presence of nicotinic acid and magnesium sulfate the BvgA protein is present only in its nonphosphorylated form (35, 36). Our data and results of Manetti et al.
suggested that in *B. pertussis* the BvgAS system is less sensitive to the modulatory effect of low temperature when compared with chemical modulators. To verify this observation, Tohama I cells were pregrown at 37 °C and then divided into four samples that were further cultivated for 6 h at 37 °C (at A600 similar to that of the T6h sample). RT-qPCR was performed using primers specific for *cyaA*, *fhaB*, *bvgA*, and *cfa* genes, and target expression levels were normalized to that of the reference gene *rpoB*. Relative expression levels determined at time 0 (T0h) were set to 1. Error bars represent standard deviations from three RT-qPCR measurements. Two independent experiments were performed, and representative results are shown.

![Figure 5](image1.png)

**Figure 5.** RT-qPCR analysis of temperature-dependent changes in *cyaA*, *fhaB*, and *bvgA* transcript levels. A and B, total RNA was isolated from samples of *B. pertussis* (B. p.) (A) and *B. bronchiseptica* (B. b.) (B) cells precultivated at 24 °C and harvested immediately (T0h) or 2, 4, and 6 h (T2h–T6h) upon shift to 37 °C. Control samples were grown separately for 6 h at 37 °C (at A600 similar to that of the T6h sample). RT-qPCR was performed using primers specific for *cyaA*, *fhaB*, *bvgA*, and *cfa* genes, and target expression levels were normalized to that of the reference gene *rpoB*. Relative expression levels determined at time 0 (T0h) were set to 1. Error bars represent standard deviations from three RT-qPCR measurements. Two independent experiments were performed, and representative results are shown.

![Figure 6](image2.png)

**Figure 6.** The effect of temperature upshift on membrane composition and on expression and production of virulence factors in *B. pertussis* B1917 strain. A–D, *B. pertussis* (B. p.) B1917 cells were grown at 24 °C, at time 0 (T0h) culture was transferred to 37 °C, and samples were taken for lipid isolation, RT-qPCR, and Western blot analyses at the indicated time points. In parallel, control samples were taken from cultures grown separately at 37 °C (at A600 similar to that of the T6h sample). A, changes in FA composition of *B. pertussis* membranes upon the temperature upshift. Error bars represent standard deviations from three measurements of two independent experiments. B, RT-qPCR was performed using total RNA and primers specific for *cyaA*, *fhaB*, *bvgA*, and *cfa* genes. Target expression levels were normalized to that of the reference gene *rpoB*. Relative expression levels determined at time 0 (T0h) were set to 1. Error bars represent standard deviations from three RT-qPCR measurements. Two independent experiments were performed, and representative results are shown. C, samples of *B. pertussis* culture were mixed with 8 M urea (1:1) and used to determine the total AC enzyme activity (secreted into the medium plus associated with cells). Error bars represent standard deviations from three measurements. Two independent experiments were performed, and representative results are shown. D, Western blot analysis of ACT, FHA, PRN, and BvgA protein levels in samples taken before (lane 1) and after shift to 37 °C (lanes 2–5) and in a control sample (lane 6). Whole-cell lysates of *B. pertussis* cultures equivalent to 0.1 A600 unit were separated by standard SDS-PAGE and transferred to nitrocellulose membrane. Protein levels were analyzed by immunoblotting using corresponding antibodies. Relevant parts of the membranes are shown. At least two independent experiments were performed, and representative results are shown. mU, milliunits.

(37) suggested that in *B. pertussis* the BvgAS system is less sensitive to the modulatory effect of low temperature when compared with chemical modulators. To verify this observation, Tohama I cells were pregrown at 37 °C and then divided into four samples that were further cultivated for 1 h at 24 or at 37 °C in the absence (control) and presence of either 20 mM nicotinate or 50 mM magnesium sulfate. The extent of BvgA phosphorylation in collected samples was assayed using Phos-tag gels. As seen in Fig. 7B, in samples treated for 1 h with chemical modulators almost all BvgA protein was dephosphorylated;
however, samples incubated at 24 °C still contained high levels of BvgA-P.

**Discussion**

In this work, we have for the first time compared the adaptation of two closely related pathogens, B. pertussis and B. bronchiseptica, to altered growth temperatures. Although both species cause respiratory diseases and exploit similar regulatory mechanisms of sensing the environment, they differ in the host range and in the ability to survive outside of the host. Therefore, comparative analysis of these two species provides a valuable model for exploration of mechanisms required for adaptation to changing environments. Our results clearly show that there is an enormous difference in adaptation to temperature changes between human-adapted B. pertussis and environmentally adaptive B. bronchiseptica.

In contrast to B. bronchiseptica, which effectively adapts its FA composition to low temperatures by increasing the content of low-melting UFAs (DI increased from 0.27 at 37 °C to 0.59 at 24 °C and 0.67 at 16 °C), the content of UFAs in B. pertussis was increased only slightly between 0.42 and 0.54 and did not further increase at 16 °C (0.55). These data clearly indicate that adaptability of B. pertussis membrane operates in a very narrow range. It is worth noting that at 37 °C the membranes of B. pertussis contained higher amounts (42%) of UFAs than did B. bronchiseptica (27%), which possibly explains why at 24 °C the UFA content in B. pertussis (54%) did not increase to a similar extent as that in B. bronchiseptica (59%). This finding most likely contributes to the observed differences in the thermal adaptation between the two species.

Consequently, at lower temperatures, the membrane fluidity measured as DPH anisotropy remained almost unchanged in B. pertussis, whereas it was significantly increased in B. bronchiseptica. In good agreement with a previous report (38), B. pertussis cells cultured at 37 °C contained predominantly saturated palmitic and unsaturated palmitoleic acids, and this pattern did not change dramatically either at 24 or at 16 °C. The FA repertoire of B. bronchiseptica membrane was wider as it in addition included CFA and oleic acids. Importantly, bacteria use FA diversity as a tool to alter their membranes in response to environmental stress (39). Among the fatty acids present in B. bronchiseptica membrane, the palmitoleic acid has the lowest melting point (0.1 °C), whereas the melting points of cyclopropane and oleic acids, which are synthesized from palmitoleic acid, are around 16 °C (40, 41). Thus, the ability to modify the content of cyclopropane, oleic, and palmitoleic acids provides B. bronchiseptica cells with a highly operative instrument allowing swift adjustment of the membrane fluidity and optimal response to thermal changes. The cyclopropanation reaction (methylation of palmitoleic acid) in B. bronchiseptica is catalyzed by cyclopropane fatty acid synthase encoded by the cfa gene (BB3369) (29). In support, we observed that elevated content of CFA in the membranes of B. bronchiseptica upon temperature upshift correlates with increased expression of the cfa gene (Fig. 5A). The lack of CFA in extracted lipids of B. pertussis motivated us to search for a cfa homologue in B. pertussis. Interestingly, we discovered that a cfa homologue in B. pertussis (BP2053) is a pseudogene. This observation clearly explained the absence of cyclopropane acid in B. pertussis membrane lipids. We suggest that (a) cyclopropanation of palmitoleic acid by the cfa gene is required for effective adaptation of B. bronchiseptica membranes to thermal changes and (b) the functionality of the cfa gene was lost in the process of adaptation of B. pertussis to a human host and its body temperature by a genome decay mechanism (29, 42).

Notably, in several pathogenic bacteria, the cyclopropanate acid provides protection against other forms of environmental stress including acidic and osmotic shocks (43, 44). It was shown that B. pertussis is less acid-tolerant than B. bronchiseptica (45), and this observation could be well explained by the lack of CFA production in B. pertussis.

Furthermore, our data indicated correlation between the ability of bacteria to modify the membrane composition and the capacity to produce virulence factors in response to thermal changes. B. pertussis cells grown at 24 °C did not cease the production of ACT, FHA, PRN, and BvgA proteins. In agreement with previous data (46), the levels of BvgA and PRN increased upon transition to 37 °C; however, the production of ACT and FHA did not increase substantially. In contrast, B. bronchiseptica cultures grown at 24 °C switched off the production of virulence factors completely; nevertheless, upon upshift to 37 °C, the production of ACT, FHA, PRN, and BvgA proteins was markedly increased, and after 6 h the production reached the levels observed in control cells grown at 37 °C. This increase in production was mirrored by an extensive decrease in desatura-
tion of the lipids in B. bronchiseptica membranes. The unexpected difference in production of virulence factors between B. pertussis and B. bronchiseptica at 24 °C resulted from a different extent of phosphorylation of the BvgA regulator. In contrast to B. bronchiseptica, B. pertussis cells contained significant amounts of phosphorylated BvgA protein at 24 °C. Apparently, B. pertussis cells were less sensitive to modulatory effects of the low temperature when compared with B. bronchiseptica. Likewise, the BvgAS system of B. pertussis was shown to be less sensitive to modulation by nicotinic acid or sulfate when compared with B. bronchiseptica (47). Nevertheless, although B. pertussis is less sensitive to modulation in vitro in general, our data indicate that its sensitivity to chemical modulators is still much higher than its sensitivity to low temperature. Importantly, our analysis of membrane composition and adaptation, production of virulence factors, and BvgA phosphorylation state in B. pertussis did not reveal any significant difference between the laboratory-adapted strain Tohama I and clinical isolate B1917.

Signals perceived by the periplasmic domains of BvgS kinase are transferred to the cytoplasmic kinase domain through the membrane via transmembrane helices (48). Interestingly, the kinase activity of BvgS can be turned off by disruption of specific interactions between the periplasmic and transmembrane domains (48). The linker region between the transmembrane and kinase domains of BvgS was also shown to be important for thermal sensing (37). Remarkably, both transmembrane and linker regions are the most invariable domains, which confirms their importance for BvgS functionality (18, 47). Thus, it is conceivable that temperature-induced changes in the membrane fluidity may affect the transmission of the signal to the kinase and thereby modulate the expression of virulence factors. Recently, it was shown that binding of nicotinate induces conformational changes within the periplasmic moiety of BvgS (36). Dupré et al. (36) proposed that these changes enable the transmembrane helices to adopt a distinct conformation that sets the cytoplasmic moiety of BvgS into phosphatase mode. We speculate that in B. bronchiseptica cells the adaptation of membrane to 24 °C induces conformational changes within the transmembrane domain of BvgS that are similar to those caused by nicotinate. Upon the shift to 37 °C, the membrane architecture may change in response to altered fluidity and allow the membrane helices to transmit the activating signal to the kinase domain again. In contrast, B. pertussis cells do not adjust the membrane composition to such a great extent and conceivably retain the BvgS kinase in semiactive state that permits default virulence factor production regardless of the environmental temperature. We hypothesize that continuous production of virulence factors, which are required for efficient infection and colonization of the host, represents a rational strategy for transmission of B. pertussis infection.

**Experimental procedures**

**Bacterial strains and growth conditions**

B. pertussis strains Tohama I and B1917 and B. bronchiseptica strain RB50 were grown on Bordet-Gengou agar plates supplemented with 15% defibrinated sheep blood at 37 °C. For liquid cultures, bacteria were grown in Stainer-Scholte medium (49) supplemented with 0.1% cyclodextrin (Sigma-Aldrich, catalogue number 332593) and 0.5% casamino acids (Difco) at temperatures indicated in the figure legends. For lipid isolation and for determination of expression and production of virulence factors, the samples were taken from cultures grown to midexponential phase (A_{600} ≈ 0.8). For growth at lower temperatures, the B. pertussis cultures were precultured at 37 °C and then further passaged at 24 or 16 °C to achieve at least three cell division generations. For temperature upshift experiments, the cultures of B. pertussis were preincubated for at least 48 h at 24 °C prior to shift to 37 °C. B. bronchiseptica cultures were grown at 37, 24, and 16 °C without preincubation. For temperature upshift experiments, B. bronchiseptica cells were preincubated for 18 h at 24 °C.

**Determination of ACT production in Bordetella cultures**

For determination of the total AC enzyme activity produced by Bordetella cells, an aliquot of the culture (100 μl) was directly mixed with 8 mM urea (1:1, v/v). AC activities were measured in the presence of 1 μM calmodulin as described previously (50). One unit of AC activity corresponds to 1 μmol of cAMP formed per min at 30 °C and pH 8.0. Enzyme activities in samples were normalized to the A_{600} of the culture.

**Lipid isolation**

Fatty acid analysis and fluorescence anisotropy measurement were performed with isolated whole-cell lipids extracted with chloroform and methanol essentially as described by Bligh and Dyer (51). Briefly, the pelleted cells were washed with 60 mM phosphate buffer (pH 7.4) and extracted with an appropriate volume of chloroform-methanol mixture (2:1, v/v) for 2 h. Cells were then separated by centrifugation (3200 × g, 10min), additional chloroform and distilled water were added to the collected supernatant, and the phases were allowed to separate for 12 h at 4 °C. The upper methanol-water phase was discarded, and the lower chloroform phase containing lipids was transferred to a clean tube and concentrated under a flow of nitrogen. The lipid isolates were stored at −70 °C.

**Fluorescence anisotropy measurement**

For fluorescence anisotropy measurement, lipids were dissolved in chloroform and then evaporated by a flow of nitrogen to form a thin layer on the walls of a glass tube. Lipids were hydrated in 50 mM Tris-HCl buffer (pH 7.0) to a final concentration of 50 μM. The sample was then incubated for 30 min at 37 °C in the dark to let the fluorescence probe incorporate into the lipid vesicles. Steady-state fluorescence anisotropy measurements were then performed in a 1 × 1-cm quartz cuvette with a FluoroMax-3 spectrofluorometer (Jobin Yvon Horiba, France) equipped with DataMax software and polarization accessory with excitation and emission wavelengths set to 360 and 430 nm, respectively. The measurement was performed under controlled tempera-
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ture conditions within the range of 8–44 °C; the temperature of the sample was controlled and monitored directly in the cuvette. The background fluorescence of non-labeled samples did not exceed 2% of the experimental values. The steady-state anisotropy was calculated as already described (54).

Fatty acid analysis

Membrane lipids were trans-esterified to fatty acid methyl esters by incubation in sodium methoxide at room temperature (55). After neutralization by the addition of methanolic HCl, the fatty acid methyl esters (FAMEs) were extracted three times with 200 μl of pentane and dried under a flow of nitrogen. Methyl esters were dissolved in 100 μl of heptane and analyzed by gas chromatography–mass spectrometry (GC-MS) using a Shimadzu GC17A–QP5050A gas chromatograph–mass spectrometer apparatus in a DB-5ms column (Agilent Technologies; 30 m × 0.25 mm; stationary phase thickness, 0.25 μm). The linear velocity of helium was set at 30 cm/s⁻¹. Sample (1 μl) was injected using an AOC-20i automatic sampler (Shimadzu) at an injection temperature of 250 °C in the split (1:40) mode. The following operating conditions were used: initial oven temperature of 70 °C for 4 min, rising at 25 °C/min to 250 °C, and held isothermally for 5 min at 250 °C. The FAMEs were identified with the aid of FAME standards (Sigma-Aldrich), and the identity was confirmed using the National Institute of Standards and Technology Mass Spectral Library (2011). The percentage of each FA was calculated by the ratio of peak area/sum of total identified peak areas.

Thin-layer chromatography

Phospholipids were separated by thin-layer chromatography (TLC; silica gel 60 G plates, Merck) in chloroform–methanol-water (65:25:4, v/v/v) as the mobile phase. The spots were detected with iodine vapor, and to localize the amino groups, the plates were treated with a 0.2% ninhydrin solution (butanol–water–acetic acid, 95:5, v/v/v). The spots were collected from the plates, digested with perchloric acid (180 °C, 20 min), and then quantified spectrophotometrically as described previously (56).

Immunoblotting

Cells from 1-ml culture aliquots were lysed by sonication and mixed with sample buffer. Whole-cell lysates equivalent to 0.1 A₄₅₀₀ unit were separated by standard SDS-PAGE and transferred to nitrocellulose membrane. Separation of phosphorylated and unphosphorylated forms of BvgA was performed essentially as described by Boulanger et al. (35) using SuperSep Phos-tag 12.5% gels (Wako Chemicals). Purified BvgA protein (kindly provided by Qing Chen) was phosphorylated in vitro according to protocol described previously (35). Following the transfer, membranes were probed with polyclonal antibodies raised against pertactin (kindly provided by Nicole Guiso), ACT, and FHA and monoclonal anti-BvgA antibodies (kindly provided by Rudi Antoine and Qing Chen) followed by incubation with secondary anti-mouse (pertactin and BvgA) or antirabbit (ACT and FHA) IgG conjugated with horseradish peroxidase. The antibody-antigen complexes were visualized using SuperSignal West Femto chemiluminescent substrate (Thermo) according to a standard protocol.

RT-qPCR

RT-qPCR analysis of cyaA, fhaB, bvgA, and cfa transcript levels was performed as described earlier (57). Briefly, bacterial pellets were lysed, and total RNA was extracted using TRI Reagent solution (Molecular Research Center) including a treatment with DNase I (Promega). Duplicates of 1 μg of total isolated RNA were reverse transcribed into cDNA following the manufacturer’s instructions in a 25-μl reaction using a reverse transcription system (Promega). RT-qPCR was performed on a Bio-Rad CFX96 instrument using SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma-Aldrich), 200 nmol of each primer, and 40 ng of reverse transcribed RNA in a 20-μl qPCR. The rpoB gene was used as the reference gene, and relative gene expression was quantified using amplification efficiency values (58).

Author contributions—B. V. conceived and coordinated the study and wrote the paper. I. K. and R. F. conceived and coordinated the study. J. B. designed, performed, and analyzed the experiments shown in Figs. 2 and 3. G. S. designed, performed, and analyzed the experiments shown in Figs. 1, 3, and 6. I. A. D. designed, performed, and analyzed the experiments shown in Fig. 5. A. D. designed, performed, and analyzed the experiments shown in Figs. 5 and 6. J. D. designed, performed, and analyzed the experiments shown in Fig. 7. J. M. performed and analyzed the experiments shown in Figs. 4 and 6. All authors reviewed the results and approved the final version of the manuscript.

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