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Yersinia enterocolitica Type III Secretion Depends on the Proton Motive Force but Not on the Flagellar Motor Components MotA and MotB

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The flagellum is believed to be the common ancestor of all type III secretion systems (TTSSs). In *Yersinia enterocolitica*, expression of the flagellar TTSS and the Ysc (Yop secretion) TTSS are inversely regulated. We therefore hypothesized that the Ysc TTSS may adopt flagellar motor components in order to use the pathogenicity-related translocon in a drill-like manner. As a prerequisite for this hypothesis, we first tested a requirement for the proton motive force by both systems using the protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Motility as well as type III-dependent secretion of Yop proteins was inhibited by CCCP. We deleted *motAB*, which resulted in an immotile phenotype. This mutant, however, secreted amounts of Yops to the supernatant comparable to those of the wild type. Translocation of Yops into host cells was also not affected by the *motAB* deletion. Virulence of the mutant was comparable to that of the wild type in the mouse oral infection model. Thus, the hypothesis that the Ysc TTSS might adopt flagellar motor components was not confirmed. The finding that, in addition to consumption of ATP, Ysc TTSS requires the proton motive force is discussed.

The plasmid-encoded type III secretion system (TTSS) of pathogenic *Yersinia* species is used to inject antihost effector proteins, called Yops (*Yersinia* outer proteins), into the cytosol of susceptible eukaryotic host cells such as macrophages. Deployment of Yops by this so-called injectisome severely disrupts cellular signalling pathways and cytoskeleton structures and this disruption leads to paralysis of the attacked cells (reviewed in references 1, 5, and 6).

Virulence-associated TTSSs have been identified in several gram-negative bacteria, but the most widely distributed TTSS is the bacterial flagellum, which is believed to be the common ancestor of all TTSSs. Accordingly, many of the TTSS structural components are conserved (18).

Among the best-conserved elements of TTSSs are their putative energizers, which share some degree of homology to α and β subunits of the F_1 component of bacterial F_0F_1 proton-translocating ATPases (18). The 48-kDa protein YscN has been identified as a possible energizer of the *Yersinia* TTSS (31). It harbors two consensus nucleotide-binding motifs, the so-called Walker boxes A and B (30). Deletion of box A has been shown to completely abolish secretion of Yops (31). However, how YscN ATPase activity is linked to the transport of Yops remains elusive.

Secretion of flagellar components required for assembly of the flagellum is believed to be driven by the ATPase FliI, a homologue of YscN. The flagellar motor, driven by the proton motive force (PMF), enables rotation of the flagellum. The motor consists of eight stators integrated into the inner membrane, each built of four MotA and two MotB subunits. Proton flow through stators is transduced into torque generated between the cytoplasmic domains of MotA and FliG, which is attached to the membrane-embedded ring of the flagellar basal body (reviewed in reference 3). Interestingly, flagellum synthesis and motility and the Ysc (Yop secretion) TTSS in *Yersinia* spp. are regulated inversely in a temperature-sensitive manner (21). *Yersinia enterocolitica* is motile at 28°C but immotile at 37°C under in vitro growth conditions due to shut down of flagellum synthesis. On the other hand, the virulence-plasmid encoded TTSS is shut down at 28°C and requires 37°C for expression. Actually, this inverse regulation is coordinated, as has recently been evidenced. Bleves et al. (4) have demonstrated that the *yop* regulon was up-regulated when the flagellum master operon was deleted. These investigators speculated that the exclusive expression of just one TTSS either circumvents interference of the homologous systems or could be explained by the usage of common components. In fact, there is evidence for the interchangeability of components among the different TTSSs (2, 9, 10, 12, 27, 32, 33), which supports the idea that simultaneous functioning of two TTSS systems could lead to interference. On the other hand, recent work from DeBord et al. (8) shows that the *ttsA* gene of *Y. enterocolitica*, encoding a membrane protein, is required for both Yop secretion and motility. This finding may support the assumption that exclusive expression of TTSSs is required to allow usage of common components.

We found it tempting to speculate that pathogenic bacteria might rotate injectisomes like a drill in order to penetrate the host cell envelope and facilitate Yop delivery. In addition, rotation of a helically assembled needle may also assist the

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movement of transport substrates through the channel. However, MotA/MotB homologues associated with TTSSs of pathogens have not been identified. Thus, we hypothesize that exclusive expression of either flagella or the Ysc TTSS in *Y. enterocolitica* may be due to the requirement of flagellar motor components by the injectisome. To test this hypothesis, we deleted *motAB* in *Y. enterocolitica* and characterized the mutant.

MATERIALS AND METHODS

Materials. The protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and the sodium motive force (SMF) inhibitors 2-*n*-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and phenamil were purchased from Sigma. Ingredients for Luria-Bertani (LB) and brain heart infusion (BHI) media were purchased from Difco.

Nucleic acid manipulations. An isogenic *motAB* mutant of *Y. enterocolitica* WA-314 (15) was constructed by the novel phage lambda Red recombinase cloning procedure (7). The entire coding regions of the *motA* and *motB* genes were replaced by a kanamycin resistance cassette. This was achieved by homologous recombination mediated by λ phage Redα and Redβ recombinases, which were expressed in *Yersinia* from a curable plasmid pKD46. For recombination, a PCR product harboring a kanamycin cassette with 50-nucleotide-homology arms was amplified from plasmid pACYC177 (New England Biolabs) with the following primers: MotABfor 5′-GGGTGCTAACGCCTCACCTTCCACCCACACACTTTGCTAAGGATATCGCGTCACTGACACCCTCATCAGTG-3′, and MotABrev 5′-GGCCGCAAAGCTTTCAGTCGCGGGTCTGATTCAATTAATTGATCCAGTTTCGTCAAGTCAGCGTAATGCTC-3′.

Transformation of this PCR product into the wild type resulted in WA-314Δ*motAB*, which was verified by PCR and a motility assay.

For complementation of WA-314Δ*motAB*, a 2.2-kb fragment encompassing the complete *motAB* genes, including the putative promotor region, was amplified by PCR with primers 5′-TCTAGAGTGCTAACGCCTCACCTTCCAC-3′ and 5′-GTCGACTACTGTGCGTCGCGGCTTG-3′, introducing XbaI and SalI restriction sites at the ends. The PCR product was ligated into vector pGEM-T (Promega). The insert was cut out with XbaI and SalI and ligated into pACYC184 (New England Biolabs) at the same sites. The resulting plasmid, p*motAB*, was transformed into WA-314Δ*motAB*.

Motility assay. Motility of *Y. enterocolitica* WA-314 and WA-314Δ*motAB* was assessed by growing strains on 0.3% floating agar (LB medium) after overnight incubation at 28°C. Optionally, agar was supplemented with CCCP (30 μM), which was added immediately before agar (45°C) was poured into petri dishes.

Analysis of secreted and translocated proteins. Yop secretion was analyzed as described previously (28). In brief, yersiniae were cultured overnight at 27°C in BHI medium. Cultures were diluted 1:40 and incubated for 2 h at 37°C in BHI medium supplemented with 200 μM $CaCl_2$. Then, Yop secretion was induced by Ca^{2+} depletion with 5 mM EGTA (16). Unless otherwise indicated, bacteria were pelleted (15 min, 10,000 × *g*) after 1.5 h of continued incubation, supernatants were precipitated with trichloroacetic acid (TCA), and precipitates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). CCCP was freshly dissolved in dimethyl sulfoxide (DMSO) and added to the cultures as indicated for the specific experiments. DMSO was added to controls not treated with CCCP to eliminate DMSO-induced side effects. The final concentration of DMSO in cultures was usually 0.5 to 1%.

Translocation of Yops was analyzed as described previously (29). HeLa cells were infected at a multiplicity of infection of 50 for 2 h. Subsequently, cells were detached by treatment with proteinase K (30 μg/ml), which was also applied to digest extracellular Yops. HeLa cells were washed twice in phosphate-buffered saline (PBS) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed by osmotic shock (resuspension in distilled H_2O with 1 mM PMSF). This treatment leaves adhering bacteria intact. Cell debris was pelleted, and the supernatant was precipitated with TCA. The precipitate was subjected to SDS-PAGE, followed by Western blotting to analyze translocated Yops.

Determination of cytosolic ATP. The ATP Bioluminescence Assay kit CLS II (Roche) was used according to the manufacturer's recommendations to analyze the influence of CCCP on intracellular ATP levels. Bacterial cultures (2 ml) were centrifuged, and the pellets were resuspended in 100 mM Tris-HCl (pH 7.75)–4 mM EDTA, with adjustment of the optical density at 600 nm (OD_{600}) of the cell suspension to 1.0. The cell suspensions (100 μl) were boiled for 2 min at 100°C. Samples were centrifuged, and 50 μl of each supernatant was transferred to a microtiter plate that was kept on ice until measurement. Luciferase reagent (50 μl) was injected, and luminescence read with a MicroLumatPlus LB 96V luminometer (Berthold Technologies) at 20°C. ATP standards provided with the kit were diluted in the range from 10^{-5} to 10^{-10} M ATP.

Immunofluorescent labeling of flagella. Bacteria grown on minimal medium (M9 minimal salts, 1% glucose, 0.1% Casamino Acids, 100 μg of thiamine/ml) agar plates overnight at 28°C were gently harvested with PBS and processed for immunofluorescent staining with the use of an anti-*Y. enterocolitica* flagellum serum and rhodamine-conjugated secondary anti-rabbit immunoglobulin (IgG) antibodies (Sigma-Aldrich, Munich, Germany). The anti-flagellum antibody was raised against *Y. enterocolitica* serogroup 0:9 flagella by immunizing a New Zealand rabbit with flagella that were purified from motile bacteria by shearing through vigorous shaking. The presence of flagella in the *Yersinia* strains was analyzed by immunofluorescence microscopy.

Mouse infection experiments. Virulence of *Y. enterocolitica* WA-314 and WA-314Δ*motAB* was tested in the mouse orogastric infection model as described previously (26). Prior to infection of 6- to 8-week-old C57BL/6 mice, *Yersinia* frozen stock suspensions (bacteria grown to late exponential phase at 27°C in LB medium) were thawed and washed twice in PBS. After appropriate dilutions, 10^8 bacteria were fed to groups of five mice with a microliter pipette. At 5 days postinfection, mice were sacrificed and the number of bacteria surviving in the lumina of the small intestine, Peyer's patches, spleens, and livers were determined.

RESULTS

PMF-dependence of Yop secretion. The *E. coli* flagellar motor is known to require the PMF (24), whereas other flagellar motors, such as that of *Vibrio cholerae*, are driven by SMF (14). Since *Yersinia* MotA and MotB exhibit high degrees of homology to *Escherichia coli* MotA and MotB (73 and 80% identity, respectively), it was expected that the *Yersinia* flagellar motor would require PMF. If MotAB engagement for Yop delivery would hold, then both motility and Yop delivery should be sensitive to the protonophore CCCP. Therefore, motility of *Y. enterocolitica* WA-314 was analyzed on floating agar in the absence or presence of 30 μM CCCP. As expected, motility was inhibited by CCCP at 28°C (Fig. 1A, left panel).

Next, we wondered whether Yop secretion would also be sensitive to CCCP. Yersiniae were preincubated at 37°C in the presence of Ca^{2+} to induce expression of the TTSS. Then, CCCP was added (concentration range of 0, 1, 5, 10, and 15 μM) and simultaneously secretion of Yops was induced by addition of EGTA, which depletes the medium of Ca^{2+} ions (Fig. 2A). Alternatively, cultures were preincubated with CCCP for 10 min prior to induction of Yop secretion (Fig. 2B). Accumulation of Yops in the culture supernatant was analyzed by SDS-PAGE 2 h after induction of Yop secretion. As can be seen in Fig. 2A, 10 μM CCCP is sufficient to completely abolish Yop secretion immediately. In order to allow a better assessment of the specificity of this phenomenon, we analyzed the influence of CCCP on growth restriction (Fig. 3A), Yop secretion and expression (Fig. 3B), and ATP level (Fig. 3C), in parallel. Yersiniae (six cultures in parallel) were preincubated in the presence of Ca^{2+} as described above. At an OD_{600} of approximately 1.0, CCCP (10 μM) was added to three cultures. DMSO, the solvent for CCCP, was added to the other three cultures at a final concentration of 0.5%. Five minutes later, secretion of Yops was induced in all six cultures by the addition of 5 mM EGTA and 10 mM $MgCl_2$. Before addition of CCCP-DMSO, 30 and 60 min thereafter, 2-ml aliquots of each culture were removed for analysis of the supernatant for Yop secretion and the pellet for intracellular Yops and ATP levels. Treatment with 10 μM CCCP caused an almost immediate growth arrest (Fig. 3A), accompanied by a complete inhibition of Yop

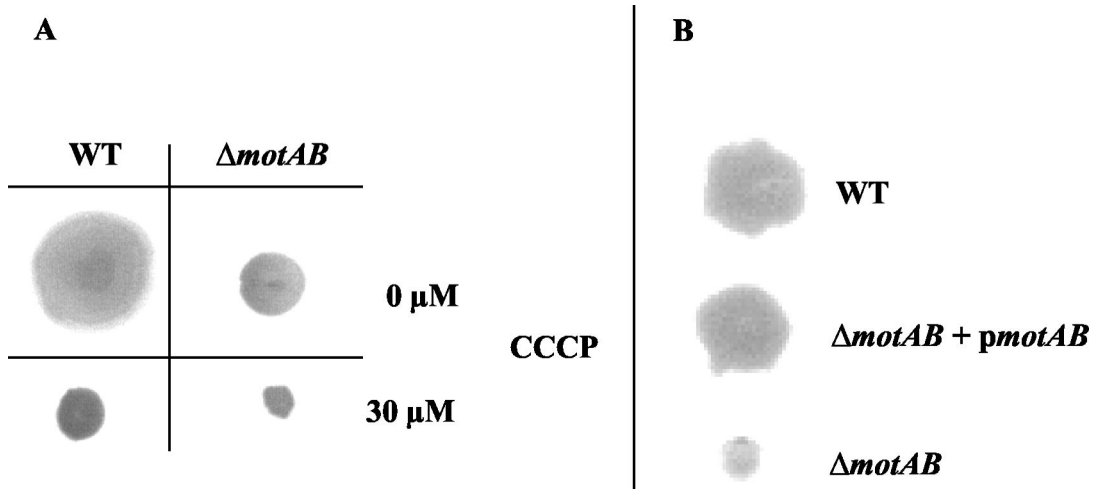


FIG. 1. Motility of *Y. enterocolitica* WA-314 (wild type [WT]) and WA-314Δ*motAB* on floating agar. (A) Yersiniae were grown on 0.3% agar (LB medium) with or without 30 μM CCCP and incubated overnight at 28°C. (B) Transcomplementation of WA-314Δ*motAB* with plasmid p*motAB* and influence on motility.

secretion (Fig. 3B, upper panel). The intracellular level of Yops, as exemplified by YopE and YopH (Fig. 3B, lower panel), did not decrease dramatically with CCCP treatment. Thus, the inhibition of Yop secretion cannot be explained by a lack of secretion substrates. Since *Yersinia* TTSS function requires ATPase activity (31), a lack of ATP after collapse of the proton gradient could account for the abolished secretion. We therefore analyzed intracellular ATP levels by a luciferase assay (Fig. 3C). We found that the ATP levels of yersiniae treated with CCCP were comparable to those of untreated cells within an hour. Hence, CCCP treatment was not accompanied by a sudden decrease in intracellular ATP. We observed a 10 to 15% decrease in ATP levels upon CCCP treatment within minutes when CCCP was added to early- or late-logarithmic cultures (OD_{600} of <0.8 or >1.4; data not shown), indicating a dependence of this reaction on the fitness of the cultures. Vigorous shaking of the cultures was also crucial for maintaining ATP levels of CCCP-treated cells comparable to ATP levels of untreated cells. Altogether, from these experiments we can conclude that Yop secretion requires PMF.

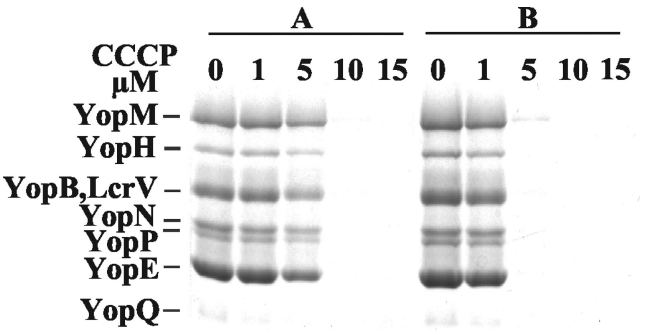
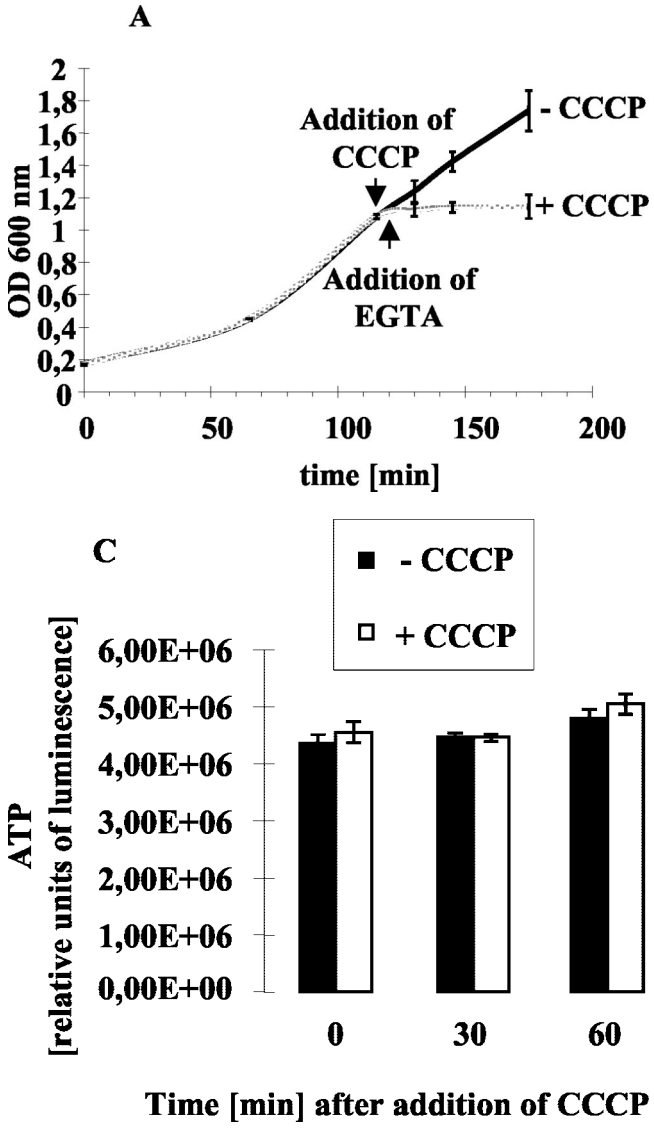


FIG. 2. *Y. enterocolitica* type III secretion is sensitive to the protonophore CCCP. (A) Yersiniae were grown at 37°C for 2 h. Then, secretion of Yops was started by complexing Ca^{2+} with EGTA; simultaneously, CCCP was added (B) Alternatively, yersiniae were grown at 37°C for 2 h, preincubated with CCCP for 10 min, and subsequently stimulated with EGTA. Secretion was analyzed after 2 h of EGTA stimulation by TCA precipitation of the culture supernatant and subsequent SDS-PAGE (Coomassie staining).

It is known that acetate can cross the membrane in its protonated form thereby acidifying the cytoplasm and accordingly reducing ΔpH but not membrane potential Δψ. In addition, it was shown that treatment of *E. coli* with acetate (34 mM and pH 5) did not dramatically decrease the total PMF but reduced motility effectively (25). We found that Yop secretion was abolished upon pretreatment with 30 mM potassium acetate (pH 5.1) (data not shown). This suggests that it is the ΔpH component of the PMF which is required for Yop secretion.

Motility and Yop secretion of *Y. enterocolitica* were not affected by substances that inhibit SMF driven motors (data not shown), such as HQNO and phenamil (14).

Yop secretion and mouse virulence are independent of MotAB. To test whether PMF for Yop secretion is deployed via the flagellar motor, we constructed an isogenic deletion mutant of *Y. enterocolitica* lacking the motor components MotA and MotB. This was accomplished by a one-step inactivation procedure based on homologous recombination between a PCR product and the target gene. Recombination by just 50-nucleotide-homology arms was made possible by expressing λ phage recombinases from a curable plasmid in *Yersinia*. Successful mutagenesis was asserted by testing motility on floating agar (Fig. 1A, right panel) and by PCR (data not shown). The *motAB* mutant was successfully complemented in *trans* by using plasmid p*motAB*, a construct based on the moderate-copy-number vector pACYC184 (Fig. 1B). To ensure that the immotile phenotype was not due to a lack of flagella, caused, e.g., by interference of mutagenesis with the flagellar regulatory network, we visualized flagella. Immunofluorescence microscopy of antiserum against flagella revealed that deletion of *motAB* did not abolish flagellar synthesis (Fig. 4). Thus, assembly of flagella that is based on flagellar TTSS secretion does not depend on the presence of the motor components MotAB. Consistent with this finding, it has been



shown that secretion of Yp1A by the *Yersinia* flagellar TTSS was not affected in a *motA* mutant (33).

Next, the influence of the *motAB* mutation on Yop secretion and translocation, was analyzed. Figure 5 shows that the *motAB*

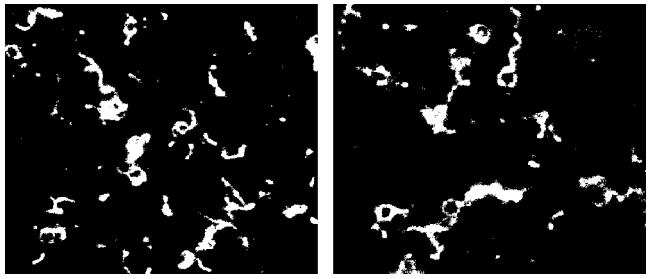


FIG. 4. Immunofluorescence microscopy of yersinia (wild-type strain WA-314 left) and mutant WA-314Δ*motAB* (right) flagella. Yersiniae were grown on minimal medium agar plates overnight at 28°C, gently harvested with PBS, and processed for immunofluorescent staining with anti-*Y. enterocolitica* flagellum serum and rhodamine-conjugated secondary anti-rabbit IgG antibodies.

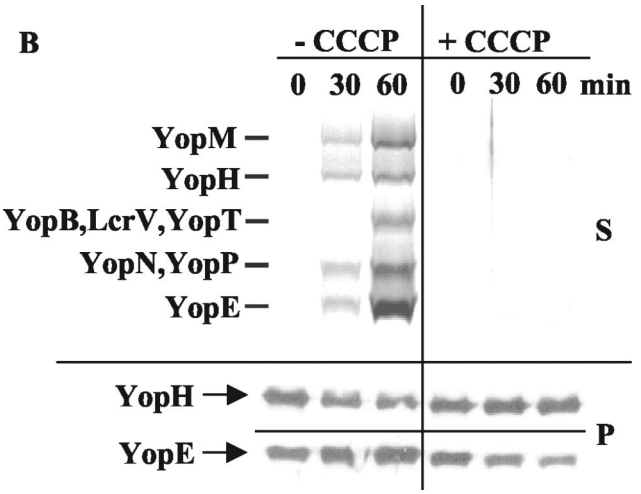


FIG. 3. Influence of CCCP on *Y. enterocolitica* growth (A), Yop secretion and expression (B), and ATP level (C). Six *Yersinia* cultures were grown in parallel for 2 h at 37°C in BHI. CCCP (10 μM) was added to three cultures, and DMSO (solvent for CCCP) was added to the other three cultures as a control. After 5 min, Yop secretion was induced by the addition of 5 mM EGTA and 10 mM $MgCl_2$. Samples of supernatant and cell pellets were taken at 0, 30, and 60 min relative to the addition of CCCP or DMSO. The OD_{600} of cultures was monitored. (B) Culture samples with OD of >1 were diluted. Supernatant was precipitated with TCA, and equal amounts adjusted by OD (corresponding to approximately 1 ml of 1 OD_{600}) were loaded onto an SDS gel and stained with Coomassie (upper panel). One fifth of corresponding cell pellets was loaded onto an SDS gel, electroblotted, and immunostained by using anti-YopE and anti-YopH antisera (lower panel). (C) For determination of intracellular ATP levels, 2 ml of bacterial cultures was centrifuged, and pellets were resuspended in 100 mM Tris-HCl (pH 7.75)–4 mM EDTA with adjustment of the OD_{600} of the cell suspension to 1.0. The cell suspensions (100 μl) were boiled for 2 min at 100°C. Samples were centrifuged, and 50 μl of each supernatant was transferred to a microtiter plate. Luciferase reagent (50 μl) was injected, and luminescence read by a luminometer at 20°C.

mutant secreted amounts of *Yops* to the culture supernatant similar to those of the wild type. The amount of intracellular Yops was also not affected (data not shown). Furthermore, no differences in cytopathic effects on infected HeLa cells or translocation

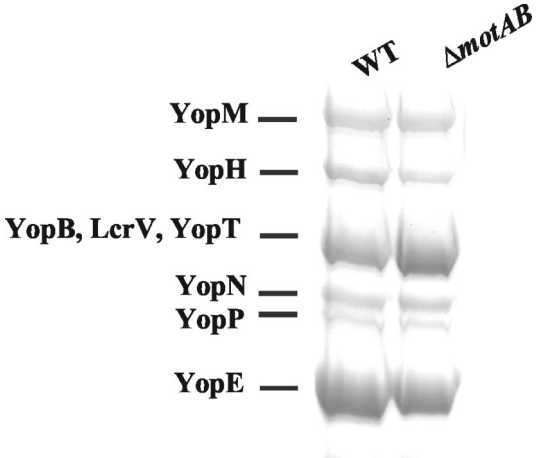


FIG. 5. Yop secretion of the *Yersinia* Δ*motAB* mutant. Secretion of Yops by the wild-type strain and its *motAB* mutant was analyzed on a Coomassie-stained gel after secretion for 90 min.

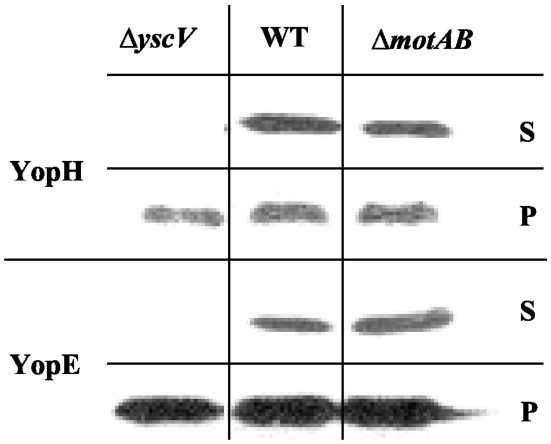


FIG. 6. Translocation of Yop effectors to HeLa cells is not affected by the deletion of *motAB*. The translocation-deficient *yscV* (*lcrD*) mutant served as a control. HeLa cells were infected at a multiplicity of infection of 50 for 2 h. Then, cells were detached by treatment with proteinase K, washed, and lysed by osmotic shock. Samples were centrifuged. S refers to supernatant after HeLa cell lysis and subsequent TCA precipitation thus corresponding to the fraction containing translocated Yops. P refers to the pellet fraction after HeLa cell lysis including adherent bacteria and cell debris. Fractions were loaded on an SDS gel, which was electroblotted. YopE and YopH were detected with appropriate antisera.

of YopE and YopH into HeLa cells as determined by Western blotting of intracellular fractions could be detected (Fig. 6).

To rule out that a possible interaction of MotAB with the injectisome could become manifest only in vivo, we orally infected C57BL/6 mice with 10^8 CFU of the *motAB* mutant or the wild-type strain. Five days later, mice were sacrificed and colony counts of surviving bacteria in organs were determined. The course of infection with the mutant was progressive, with bacteria colonizing not only the small intestine and Peyer's patches but also the spleen and liver, as is the case for the wild-type strain. No significant difference in bacterial colony counts between the wild type and the mutant could be detected in Peyer's patches (5.42 ± 1.13 and 5.58 ± 0.73 mean log CFU $\pm$ standard deviations, respectively) and the spleen (6.43 ± 0.79 and 6.21 ± 1.22 mean log CFU, respectively). In summary, no evidence was found that MotAB function is linked to type III-dependent transport of Yops.

DISCUSSION

In this study we examined whether the flagellar motor components MotA and MotB and the PMF play a role in protein secretion of Yops by the *Y. enterocolitica* TTSS. We could demonstrate that a *motAB*-deficient *Yersinia* mutant was fully virulent in the mouse orogastric infection model and competent to secrete and translocate Yops. Interestingly, additional insights can be gained from this study. Flagellum-dependent motility is essential for virulence of many pathogenic bacteria (for a review, see reference 20). However, the role that motility plays in the pathogenicity of *Yersinia* is less clear. The invasion capacity of an immotile *Y. enterocolitica fliA* mutant was shown to be unaffected in the mouse model after intragastric infection (19). Further, it was demonstrated that an immotile *Y. enterocolitica flgM* mutant was fully virulent according to the 50% lethal dose after intragastric inoculation (22). This finding seems reasonable since flagellar synthesis should be repressed in a 37°C host environment. However, Young et al. (34) later demonstrated in cell culture experiments that invasin-mediated invasion at 27°C requires flagellum-dependent motility to enable yersiniae to efficiently reach susceptible host cells. It is not known whether yersiniae are still motile after oral uptake by the time they have reached the M cells overlying Peyer's patches of the ileum. Therefore, it cannot be judged definitively whether motility plays a role in vivo during the infection process. However, we found no evidence for an influence of flagellum-dependent motility on dissemination of yersiniae to Peyer's patches, the spleen, and the liver, suggesting that motility is dispensable for successful infection.

The mode of energy transduction that allows type III-dependent transport of proteins is largely unexplained. Several TTSS ATPases, such as *Yersinia* YscN (31), have been identified, but their mode of action remains elusive.

In this study, we have shown for the first time that the *Y. enterocolitica* TTSS requires the PMF for secretion of Yops in addition to YscN (31). How can the PMF be used for export? The cytoplasmic side of the membrane is negatively charged relative to the periplasmic space. Accordingly, transport of negatively charged secretion substrates could be driven by the membrane potential $\Delta\psi$. Since the pI of secretion substrates of the *Y. enterocolitica* TTSS ranges from 4.44 to 9.79, a simple electrophoretic model cannot be assumed for all secretion substrates. A dominant role of $\Delta\psi$ is also not supported by our finding that acetate, which decreases only ΔpH (25), can abolish Yop secretion completely.

A recent phylogenetic analysis of P-loop NTPases by Lupas and Martin (23) in conjunction with a preceding study by Gorbalenya and Koonin (13) suggests that TTSS ATPases belong to the group of unfoldases. We therefore propose that after substrate unfolding mediated by YscN, possibly accompanied by pushing the unravelled polypeptide chain into the transport channel, additional driving force for protein export is generated by coupling to the PMF. We found that dependency of flagellar growth on the PMF was described (11 and references therein) long before the common principle of the flagellum and other TTSS machineries was identified. In addition, Galperin et al. (11) demonstrated the posttranslational assembly of flagella, recently confirmed by Hirano et al. (17). This finding supports our view that posttranslational secretion and the requirement of unfolding of TTSS substrates on the one hand and the use of PMF as a driving force on the other hand are common features of TTSSs.

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